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A case of autochthonous human Dirofilaria infection, Germany, March 2014

D Tappe (tappe@bnitm.de)[1], M Plauth[2], T Bauer[3], B Muntau[1], L Dießel[4], E Tannich[1,5], P Herrmann-Trost[4]
1. Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany
2. Städtisches Klinikum Dessau, Klinik für Innere Medizin, Dessau-Roßlau, Germany
3. Mund-Kiefer-Gesichtschirurgie Halle Dessau, Dessau, Germany
4. Amedes MVZ für Pathologie und Zytodiagnostik Halle/Saale, Germany
5. German Centre for Infection Research, partner site Hamburg-Luebeck-Borstel, Hamburg, Germany

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In March 2014, an infection with the nematode *Dirofilaria repens* was diagnosed in a German citizen in the federal state of Saxony-Anhalt. The patient had developed an itching subcutaneous nodule containing a female worm, which was identified as *D. repens* by 12S ribosomal ribonucleic acid (rRNA) gene sequencing. Autochthonous human *D. repens* infections have not been described in Germany so far, but this finding is consistent with the recent detection of *D. repens* in mosquitoes from east Germany.

Here we report the clinical and laboratory findings of the first autochthonous *Dirofilaria* infection acquired in Germany, diagnosed in early 2014.

Case description

A previously healthy German citizen aged in the late thirties was seen at a maxillofacial surgery centre in Dessau (federal state of Saxony-Anhalt), Germany, on 26 February 2014, with a subcutaneous nodule on the right temple. The lesion had developed over the past four weeks and was accompanied by an itching sensation and occasional stabbing pain with increasing intensity. The patient did not report fever or any other symptoms. On examination, a discrete pea-sized skin nodule without surrounding inflammation was found, which was excised under local anaesthesia.

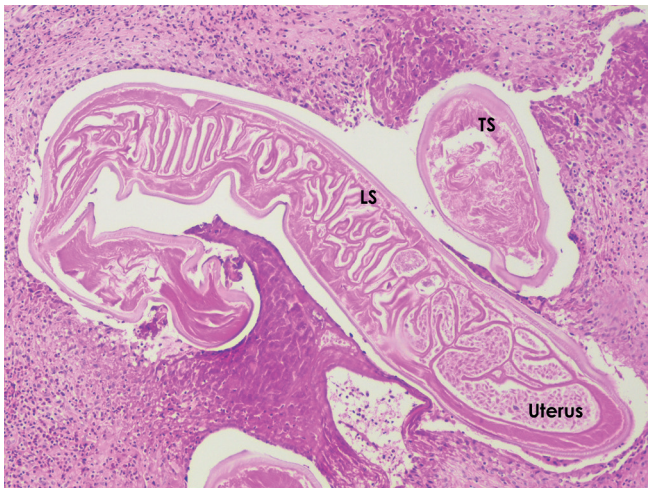
Investigation of the cause of infection

Histopathological examination of the excised 8 × 4 × 3 mm nodule revealed yellowish tissue with multiple longitudinal and transverse sections through a nematode (Figure 1). The organism was surrounded by a dense inflammatory infiltrate, which contained eosinophils, macrophages, epitheloid cells, lymphocytes, and a few multinucleated giant cells. The nematode's cuticle showed external ridges suggesting a *Dirofilaria* infection. Internal structures, like a folded paired uterus and a digestive tract were clearly visible.

A nematode-specific 12S ribosomal ribonucleic acid (rRNA) gene-polymerase chain reaction (PCR) [1] from the formalin-fixed paraffin-embedded specimen was positive. Sequence analysis of the 510 bp amplicon (www. http://blast.ncbi.nlm.nih.gov), revealed 99% similarity with *D. repens* sequences isolated in Turkey and Italy (GenBank accession numbers: KC953031, and AJ544832, AM779773, respectively). A serum sample drawn on 17 March 2014 showed a titre of 42 U (normal value <10) in an enzyme-linked immunosorbent assay (ELISA) using a crude *Dirofilaria immitis* antigen extract. Other ELISAs employing crude *Ascaris lumbricoides* and *Strongyloides stercoralis* antigens were negative. The patient was not treated with anthelminthics.

Figure 1

Longitudinal and transversal sections through female *Dirofilaria repens* nematode recovered from a patient in Germany, March 2014



LS: longitudinal section; TS: transversal section.
Haematoxylin and eosin stain, original magnification ×100.
The extensively folded uterus is clearly visible in the longitudinal section.

Follow-up of the patient

Fourteen days after the operation, the patient was referred for further diagnostic work-up. On physical examination, cardio-pulmonary, abdominal and neurological status was normal. A chest X-ray and abdominal ultrasound revealed no pathology. Full blood count revealed 7% eosinophils (normal range <7%) and a total leukocyte count of 7.5 Gpt/L (normal range: 3.8–10.5). Erythrocyte sedimentation rate (ESR) and routine clinical laboratory tests were normal.

Investigation of possible sources of infection

During the last 37 years, the patient has been living in the state of Saxony-Anhalt, in a farm house in a small village close to the river Elbe. The patient owns a pet dog and several horses. Parasitological examination of blood drawn from the patient's dog was negative for microfilariae. Except for a one-day trip to Poland, in mid-December 2013, and a short vacation trip to the Czech Republic in winter 2012, the patient had not travelled elsewhere within the past five years. The patient is a passionate angler and used to catch fish in two nearby quarry ponds in the Bitterfeld region, Saxony-Anhalt. The patient recalled multiple mosquito bites during fishing sessions in September 2013, and while working on farm house fields. The whole area was affected by the floods from the nearby Elbe river in summer 2013, and many farm fields remained partially flooded for a prolonged time.

Background

D. repens is a filarial nematode of dogs and other carnivores as definitive hosts. Similar to other filarial species, microfilariae of *D. repens* are transmitted by mosquitoes, and fertile macrofilariae develop in the natural definitive host. Humans may become infected as abberant hosts and with a few exceptions, the worm remains infertile, and therefore microfilariae are not produced [2]. The incubation time in human infections is not well defined but has been estimated to range between four and eight months [3].

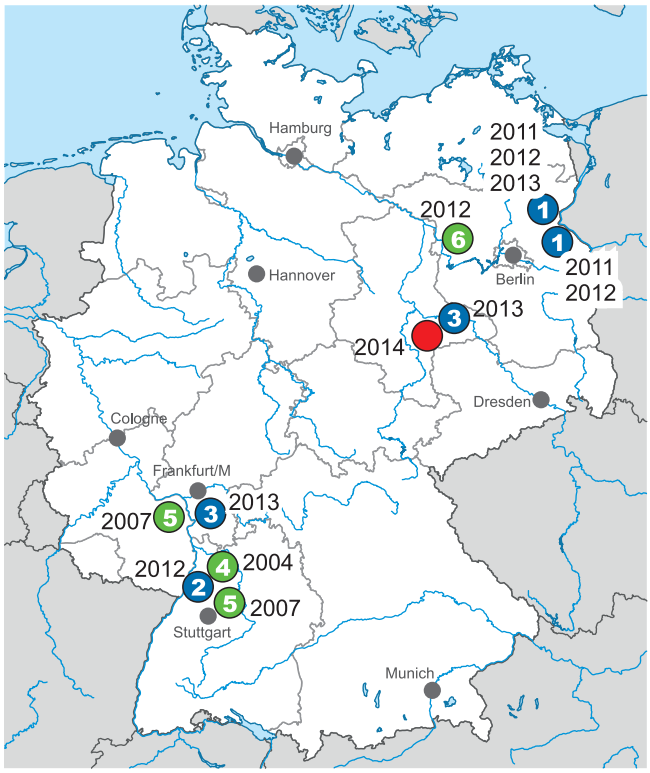
Clinically, a single subcutaneous nodule is most often present [4,5]. Local swellings with changing localisations, caused by migrating worms, may occur [6]. Only rarely, cases of organ infection have been described, affecting the lungs, genitals, breasts, or the eye [4,5]. Recently, a severe meningoencephalitic infection in a traveller returning from India and Sri Lanka has been described and the capacity of filariae to cause life-threatening disease has been demonstrated [6].

Diagnosis may be achieved parasitologically, when a living and intact worm can be extracted and inspected, or histopathologically, after surgical excision of the infected tissue. As there are numerous and morphologically very similar zoonotic *Dirofilaria* species, molecular identification of the organism should be aimed for [6]. Eosinophilia and positive *Dirofilaria* serology may be present [6]. In infections affecting the skin only, surgical removal of the parasite is effective and usually no further treatment is required. In generalised infections, however, additional systemic therapy with albendazole is recommended [6].

Human *Dirofilaria* infections have been reported in Africa, Asia and also in Europe where dirofilariasis is an emerging zoonosis [7]. The areas in Europe where endemicity of *D. repens* has been solidly established, concern countries of the Mediterranean region [7], where the warmer climate facilitates the development of infectious larvae in mosquitoes. However, during the past decade, several sporadic autochthonous human and canine cases of dirofilariasis have been reported from countries further north in central Europe, including Austria, the Czech Republic, and Poland [8–10]. Until recently, central Europe, including Germany, was not considered a region in which *D. repens* is endemic. However, the recent finding of *D. repens*-infected dogs and mosquitoes in east Germany in the federal state of

Figure 2

Geographical distribution of autochthonous human and canine *Dirofilaria repens* infections and origin of mosquitoes testing positive for *D. repens* DNA, Germany, as of March 2014



Numbers within the circles refer to references. 1: Czajka et al. 2014 [11]; 2: Kronfeld et al. 2014 [13]; 3: Tannich, unpublished observation; 4: Hermosilla et al. 2006 [14]; 5: Panchev et al. 2009 [15]; 6: Sassnau et al. 2013 [12]. The years of detection are indicated beside each coloured circle.

Brandenburg (which is located next to Saxony-Anhalt) [11,12], as well as infected mosquitoes in southwest Germany [13] suggested that stable transmission of *D. repens* is already taking place in Germany as the northernmost transmission area in Europe so far [11,12]. The present case of an autochthonous human infection in east Germany lends further support to this notion.

Discussion and conclusions

This is the first report of a laboratory-confirmed autochthonous human *Dirofilaria* infection in Germany. Infected mosquito populations have recently been detected in Saxony-Anhalt (data not shown), close to where the patient resides. The case highlights that shortly after the repeated detection of the parasite in local mammalophilic mosquito populations, human infections may occur (Figure 2).

The case's dog was not infected and thus not the reservoir of the worm. The geographical location where the case's infection most probably occurred was in the federal state of Saxony-Anhalt, where the patient had been affected from multiple mosquito bites on farm grounds and while fishing. The area was severely affected by the Elbe river floods, a circumstance, which may have greatly facilitated an increased transmission rate of *D. repens*. An infection of the case outside of Germany is highly unlikely as the visits to Poland (3 months prior to signs of infection) and the Czech Republic (over 1 year prior) were both in the wintertime with no mosquito activity. Moreover, the reported incubation period of four to eight months fits well with an infection in late summer 2013 in Germany, where, during this time, the case reported having been bitten by mosquitoes.

At present, the true burden of *D. repens* infections in Germany is unknown as data on parasite prevalence in both humans and dogs are not available. However, the data available stress the need for timely information of physicians to increase awareness for the disease, and veterinarians to implement control measures, such as treatment of infected dogs and other canines to reduce the parasite burden and thus the transmission likelihood.

Conflict of interest

None declared.

Authors' contributions

Wrote the manuscript: DT, TB, BM, ET, MP, LD, PHT; performed laboratory or epidemiological investigations: DT, BM, ET, LD, PHT; performed data analysis: DT, BM, ET, PHT.

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Invasive infections due to *Streptococcus pyogenes*: seasonal variation of severity and clinical characteristics, Iceland, 1975 to 2012

L B Olafsdottir[1], H Erlendsdóttir[2,3], J Melo-Cristino[4], D M Weinberger[5,6], M Ramirez[4], K G Kristinsson[2,3], M Gottfredsson[1,3]
1. Department of Medicine, Landspitali University Hospital, Reykjavik, Iceland
2. Clinical Microbiology, Landspitali University Hospital, Reykjavik, Iceland
3. Faculty of Medicine, School of Health Sciences, University of Iceland, Reykjavik, Iceland
4. Institute of Microbiology, Institute of Molecular Medicine, Faculty of Medicine, University of Lisbon, Lisbon, Portugal
5. Division of International Epidemiology and Population Studies, Fogarty International Center, National Institutes of Health, Bethesda, Maryland, United States
6. Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, Connecticut, United States

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Epidemiology and clinical characteristics of invasive Group A streptococcal infections (IGASI) are highly variable. Long-term studies are needed to understand the interplay between epidemiology and virulence. In a population-based study of IGASI in Iceland from 1975 to 2012, 288 cases were identified by positive cultures from normally sterile body sites. Charts were reviewed retrospectively and *emm*-types of viable *Streptococcus pyogenes* isolates (n=226) determined. Comparing the first and last decade of the study period, IGASI incidence increased from 1.09 to 3.96 cases per 100,000 inhabitants per year. The most common were *emm* types 1 (25%), 28 (11%) and 89 (11%); *emm*1 strains were most likely to cause severe infections. Infections in adults were significantly more likely to be severe during the seasonal peak from January to April (risk ratio: 2.36, 95% confidence interval: 1.34–4.15). Significant seasonal variability in severity was noted among patients with diagnosis of sepsis, respiratory infection and cellulitis, with 38% of severe infections in January to April compared with 16% in other months (p<0.01). A seasonal increase in severity of IGASI suggested that generalised seasonal increase in host susceptibility, rather than introduction of more virulent strains may play a role in the pathogenesis of these potentially fatal infections.

Introduction

Streptococcus pyogenes, or Lancefield group A Streptococcus (GAS), is known for its ability to cause diverse clinical manifestations, including soft tissue infections, pneumonia, and toxic shock syndrome. Over the past 30 years there has been a concern about increasing incidence and severity of invasive group A streptococcal infections (IGASI) [1-3], defined as infections associated with the isolation of the bacteria from a normally sterile body site. GAS has many virulence factors; a key factor is the M protein of the cell wall for which more than 100 different types are known [4]. Sequencing of the *emm* gene and classification of the M protein is often used to characterise GAS isolates [5] with studies from the United States (US) and Europe showing that isolates with M types 1, 3, 4, 6 and 28 cause about 50% of IGASI [6-8].

IGASI are often life-threatening; The most severe clinical presentations are necrotising fasciitis (NF), an aggressive and rapidly destructive infection of deep subcutaneous tissue and fascia, characterised by necrosis of skin and underlying structures, and streptococcal toxic shock syndrome (STSS), defined as streptococcal infection associated with sudden shock and organ failure [3,9]. Certain risk factors for invasive infections have been established such as the extremes of age, immunosuppression, diabetes mellitus and loss of skin integrity [10,11]. The factors driving the increased incidence of IGASI are not completely understood, however, clinical and microbiological studies demonstrate a complex epidemiology with great spatiotemporal variation of this common pathogen.

It is well established that the incidence of GAS infections can fluctuate in time, both seasonally and over the course of several years. However, most published studies on IGASI are limited by a relatively short observation period. Studies have shown, with remarkable congruence between countries, an interesting seasonal pattern of non-invasive and invasive infections, with the incidence being high in the months January to March but low in late summer and autumn [12,13]. A large European study showed that several *emm* types had a uniform seasonal prevalence whereas other types exhibited more fluctuations [14]. Little is known about whether severity of infection varies seasonally.

The purpose of this study was two-fold: Firstly, to provide a long term nationwide analysis of IGASI in Iceland by using clinical and population-based data to assess epidemiology, clinical characteristics, therapy and outcome. Secondly, to study seasonal variations in the incidence, severity, and clinical presentation of IGASI and the relationship to *emm* type prevalence.

Methods

Setting and case definition

We conducted a nationwide study of IGASI diagnosed in Iceland from 1 January 1975 to 31 March 2012. A case of IGASI was defined as isolation of GAS from a normally sterile body site, or isolation of the organism from a non-sterile site, in conjunction with the diagnosis of STSS or NF. Cases were identified through microbiology databases and hospital discharge diagnosis using the appropriate ICD codes (ICD-9 number (period 1983–96): 728.0; ICD-10 numbers (period 1997–2005): A48.3; M60.0; M63.0; M63.2). Using these criteria, 288 cases were identified and for these, 275 records were retrieved and reviewed retrospectively (yield: 96%), but age and vital status was known for all 288 cases. Cases were defined as severe, if their condition required admission to intensive care and/or if the patient died from the infection during hospitalisation. The study was approved by the Icelandic National Bioethics Committee, by the Data Protection Authority of Iceland, and by the responsible Chief Medical Officer at healthcare facilities where clinical data were obtained.

Data collection

Data collected included age, sex, past medical history, medication, presenting symptoms, physical findings, and laboratory results upon admission and treatment. The main antibiotic treatment was defined as the antibiotic used for at least half of the treatment duration.

Microbiology and *emm* typing

During the first 15 years of the study, a Bactec (Becton Dickinson Microbiology Systems) radiometric system was most widely used. During 1990–2001, Bactec (Becton Dickinson), ESP (Difco) and bioMérieux Vital (bioMérieux) nonradiometric systems were used. The BacT/Alert system (bioMérieux) was used from 2002 [15,16]. Invasive specimens, except blood cultures, were inoculated on blood agar and chocolate agar plates (Difco) and incubated under a humidified atmosphere containing 5% CO_2, as well as anaerobically for 24 hr. All positive blood culture bottles showing Gram-positive streptococci were inoculated in the same manner. All isolates were kept in Trypticase Soy Broth (TSB; Becton Dickinson) containing 20% glycerol and stored at –80°C. The *emm*-typing of isolates was performed according to the protocols and recommendations of the US Centers for Disease Control and Prevention (CDC) [17], and the first 240 bases of each sequence were searched against the *emm* CDC database [4]. Identity of at least 95% with previously described sequences over the first 150 bases considered allowed the assignment of an *emm* type. Sequencing was done by the Sanger method using an Applied Biosystems 3730xl DNA Analyzer. Subtyping was not performed.

Statistical analysis

The national population registry was used to calculate incidence and age-specific incidence rates (population data available at Statistic Iceland [18]). The crude in-house and 30-day mortality was assessed using hospital records and the national population registry.

Comparison of categorical variables was performed using Fisher´s exact test and Pearson´s test using SPSS, version 10.5. Level of significance was set at $p<0.05$, and all tests were two-tailed. Risk ratios or incidence rate ratios and their associated 95% confidence intervals (CI) were calculated using log-linked models (Poisson or binomial distribution, as appropriate) in PROC GENMOD (SAS v9.2). Dummy variables were used to test the risk or incidence during a given time period with the noted reference time period. We also considered whether influenza activity was associated with increased GAS incidence. To do this, we fit a Poisson regression where the outcome was monthly GAS cases, and included predictors for linear trend, monthly indicator variables for the winter months and influenza epidemic periods [19]. Influenza epidemic periods were defined as month when influenza activity was significantly above a harmonic seasonal baseline, determined using Serfling regression [20]. We included data between 1985 and 2008 for the influenza analyses.

Results

Epidemiology

From 1 January 1975 to 31 March 2012, 288 cases of IGASI were identified, including 54 children (≤16 years-old) and 234 adults. There were slightly more female (54%) than male cases. The number of cases diagnosed by six-year intervals is shown in Figure 1A. The incidence during 1975 to 1986 was 1.09 cases per 100,000 inhabitants per year and rose to 3.08 and 3.96 cases per 100.000 inhabitants per year during 1987 to 1998 and 1999 to 2010, respectively (rate ratios: 2.82; 95% CI: 1.87–4.25 and 3.63; 95% CI: 2.45–5.38). Figure 1B shows the age-specific incidence rates for both halves of the study, 1975 to 1993 and 1994 to 2012, highlighting an increase in most age groups during the second half, which is not explained by difference in age composition between study periods. The age-specific incidence was highest at both extremes of age (Figure 1B).

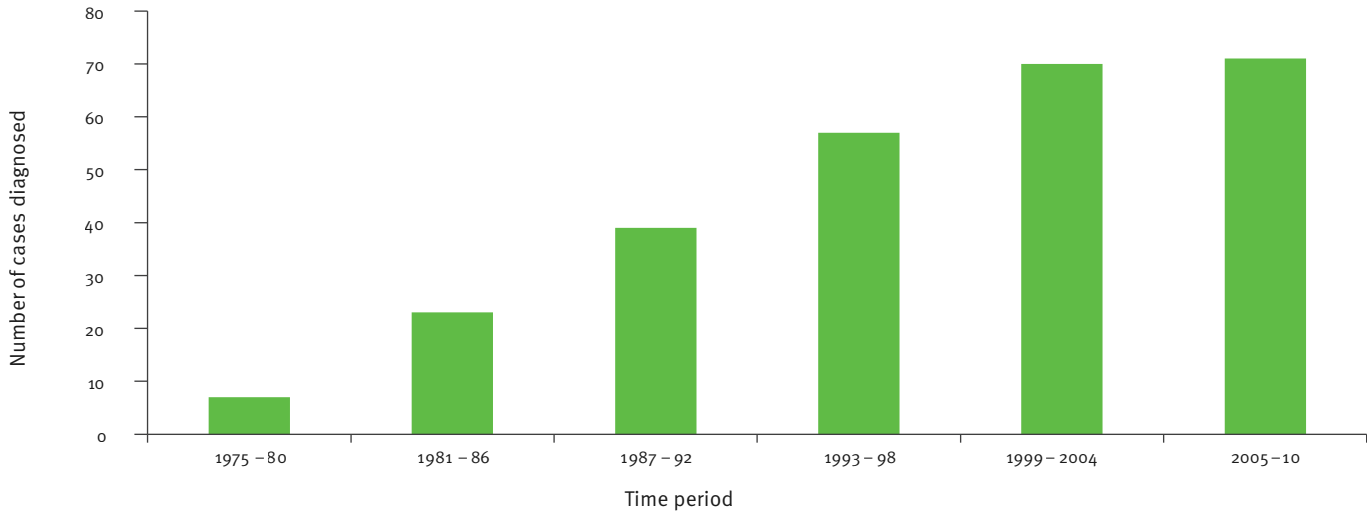
Clinical characteristics

In this retrospective study 162 of the 231 evaluable cases, had at least one underlying medical condition recorded, including cardiovascular disease in 59, malignancy in 34, respiratory disease in 28, diabetes mellitus in 12 and alcohol and/or substance abuse in 14 of the 231. In five cases, the infection was linked

Figure 1

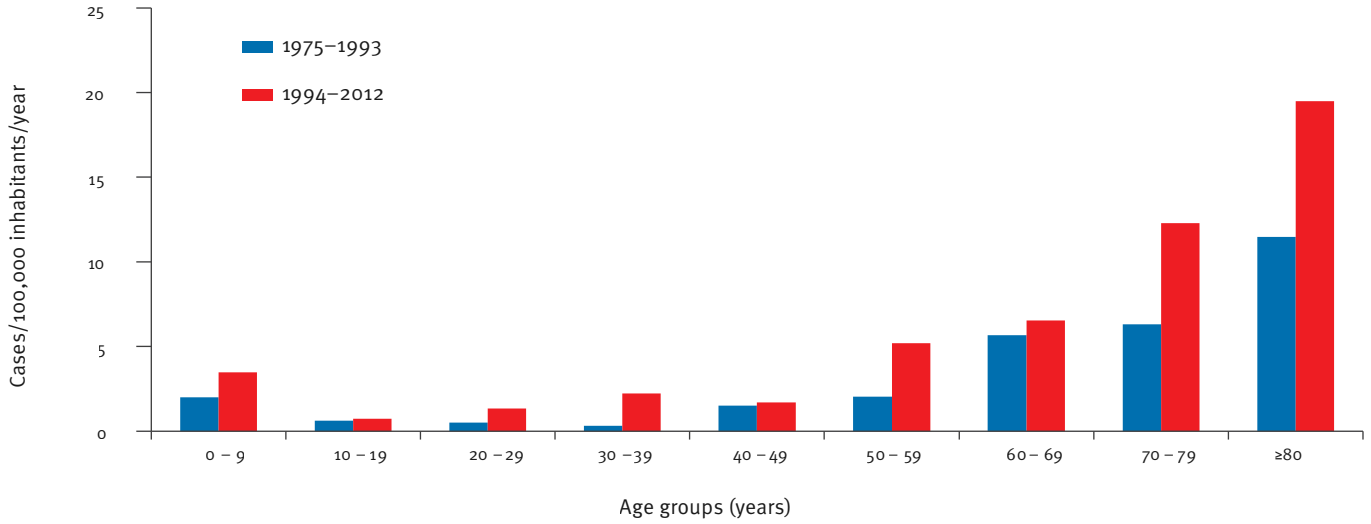
Epidemiology of invasive Group A streptococcal infection in Iceland

A. Number of diagnosed cases by six-year intervals, 1975–2010 (n=267)



The incidence during 1975–86 was 1.09 cases/100,000 inhabitants/year and rose to 3.96 cases/100,000 inhabitants/year during 1999–2010.

B. Age-specific incidence, 1975–2012 (n=288)



Blue bars: incidence rates for the first half of the study; red bars: incidence rates for second half.
The incidence almost doubled in almost all age groups.

to illicit intravenous drug use. No patient had underlying human immunodeficiency virus (HIV) infection in our cohort, whereas five children had chickenpox. Drug information prior to admission was available for 198 patients, of whom 19 were receiving immunosuppressive therapy and eight had received antibiotics before admission; there was documented non-steroidal anti-inflammatory drug (NSAID) usage prior to admission in 25 patients.

The association between age, clinical characteristics and outcome (severe and non-severe) is shown in Table 1. Skin/soft tissue infections were the most common manifestation, diagnosed in 33% of the cases, followed by sepsis in 24%. Puerperal sepsis was diagnosed in eight women and three children. STSS and NF were diagnosed in 11% of the cases, and there was an increase in the number of STSS and NF cases diagnosed during the study period, with only four cases documented from 1975 to 1994, compared with 10 cases from 1995 to 2004 and 18 cases from 2005 to 2012.

Therapy and outcome

The most common treatment was a beta-lactam antibiotic (208/219), usually penicillin. In severe infections

Table 1

Age-specific incidence per 100,000 per year, clinical syndrome and severity[a] of invasive Group A streptococcal infections in Iceland, 1975–2012 (n=288)

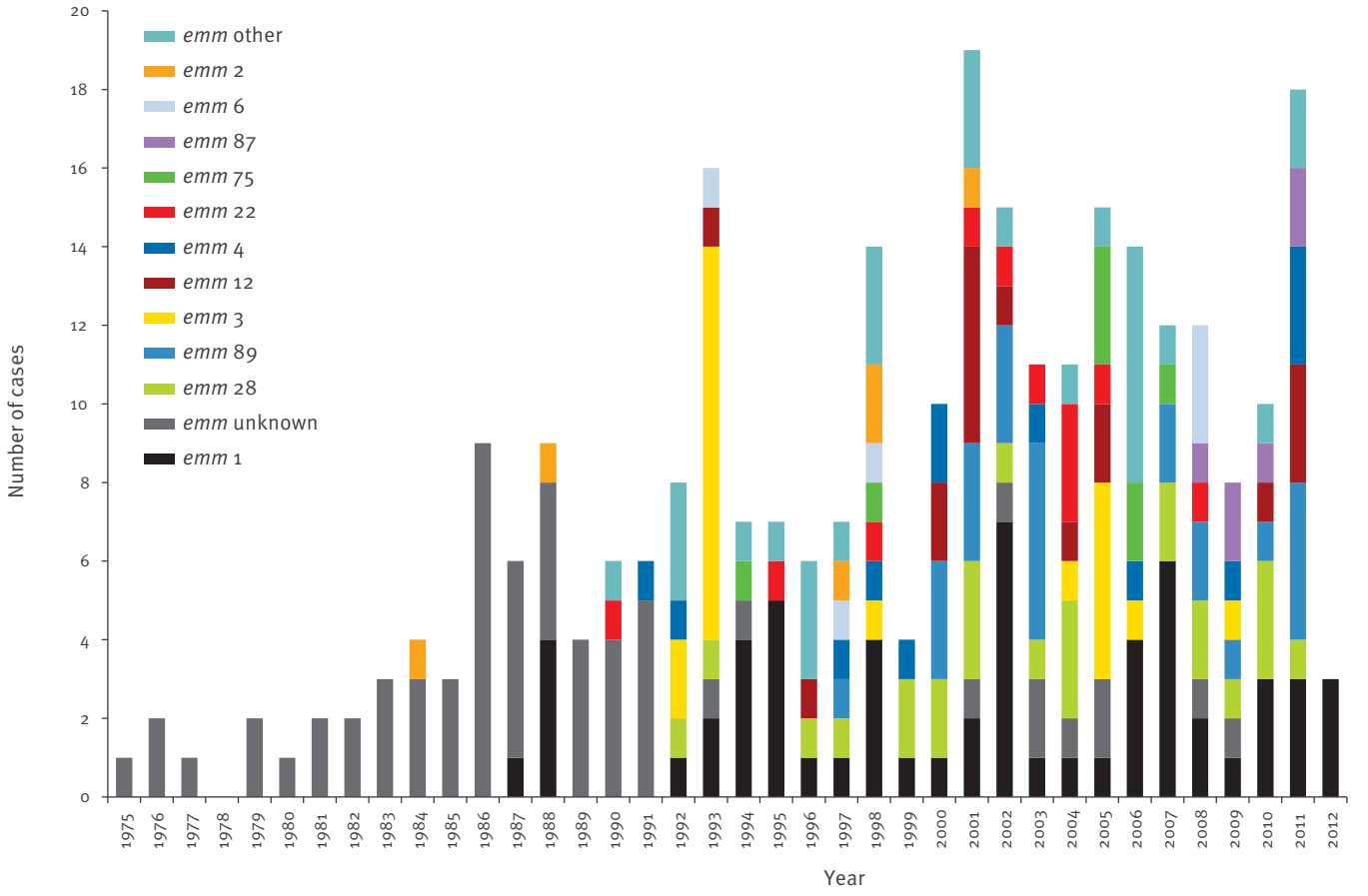
Syndrome	Age (years)								
	0–9	10–19	20–29	30–39	40–49	50–59	60–69	70–79	≥80
Arthritis	0.26	0	0.06	0.14	0.16	0.30	0.55	0.60	0.75
Skin/soft tissue	0.49	0.18	0.31	0.35	0.73	1.31	2.06	3.20	7.93
Other[a]	0.43	0	0.06	0.07	0.16	0.40	1.10	2.40	2.24
Respiratory tract infection	0.31	0.06	0	0.07	0	0.20	0.27%	1.00	2.61
Sepsis	1.22	0.12	0.31	0.42	0.40	0.71	1.37	2.20	2.20
STSS	0.06	0	0.19	0.14	0.16	0.71	0.41	0.40	0
NF	0	0	0	0.21	0	0.30	0.41	0	1.12

NF: necrotising fasciitis; STSS: streptococcal toxic shock syndrome.

[a] The category "Other" includes bacteraemia with unclear focus of infection (14 cases), osteomyelitis (five cases) and upper respiratory infection (seven cases). It also includes 18 cases for whom information other than culture data, age, sex and vital status was unknown.

Figure 2

Distribution of *emm* types during the study period, Iceland, 1975–2012 (n=226 cases)



For 2012, the first three months are included.

The *emm* types showed much fluctation in prevalence during the study period.

(n=83), other drugs were used in combination, most commonly clindamycin (27/219), followed by aminoglycosides (27/219). The average length of parenteral antibiotic treatment was 11.9 days. During the study period, 77 of the 256 patient for whom we had this information were admitted to an intensive care unit (ICU), 56 of them needed inotropic drugs, 28 required mechanical ventilation and 10 received dialysis for renal failure.

The crude 30-day mortality was 13.4% in adults and 5.6% in children. STSS or NF was associated with a significantly higher mortality rates than other infections in adults (29% vs 11%; risk ratio: 2.68; 95% CI: 1.36–5.27).

emm types

Overall 25 different *emm* types were identified among the 226 viable isolates that were recovered between 1987 and 2012. Figure 2 shows the *emm* types obtained during the study period. Table 2 shows the distribution of *emm* types in children compared with adults. The most common types were *emm*1 (25%), 28 (11%), 89 (11%), 3 (9%), 12 (8%). Among the 25 cases (10 female, 15 male), no child was diagnosed with *emm*28, and only one case of puerperal sepsis was caused by *emm*28.

The association between clinical presentation, *emm* types, and severity is summarised in Table 3. Patients infected by *emm* 1-type strains, compared with all other *emm* types identified, were more likely to have severe disease (p<0.03), whereas patients with *emm*89 had a less severe course of disease, required intensive care less frequently and had no fatalities (p=0.05). In addition, the severity differed greatly by clinical presentation, with NF (4 of 12 cases) and STSS (6 of 20 cases) being associated with a fatal outcome in 31% of cases. Invasive respiratory tract infection (4/23 cases) and sepsis (12/69 cases) were associated with a fatal outcome in 17% of cases. All cases linked to illicit intravenous drug use were caused by different *emm* types. Puerperal sepsis was caused by *emm* type 1 in six cases and *emm* types 3, 4, 28, 77, 89 in one case each. Clinical syndromes of cellulitis and arthritis were associated with least mortality, three of 95 and 0 of 22 cases, respectively.

Seasonality of incidence and severity of invasive Group A streptococcal infection

There was significant seasonal variation in the incidence of IGASI (Figure 3). The association between incidence by month and clinical presentation is given in Figure 3A, demonstrating that the diagnoses of sepsis, NF and STSS were more common in mid- to late winter with nine of 12 NF cases and eight of 20 STSS cases diagnosed during January to April. Figures 3B and 3C show the incidence by severity and month of diagnosis in children and adults, respectively. The incidence peaked in late winter and early spring, specifically in January to February for children, and in April for adults. No seasonal variation in severity was noted among children, but the group was small with relatively few fatalities. Among adults, cases of IGASI were significantly more likely to be severe (resulting in death or ICU admission) during the seasonal peak in January to April than in the period June to September (Figure 3C) (risk ratio: 2.36, 95%CI: 1.34-4.15). No association was found between IGASI and influenza epidemic period. The number of cases occurring per season in this study may be insufficient to detect such an increase above the seasonal baseline.

It is possible that the seasonal variations in severity were simply a result of differing clinical presentations during the peak months, so we examined the individual seasonal variations in severity of those with a diagnosis of sepsis, respiratory infections, or cellulitis (excluding NF and STSS patients and arthritis or other clinical manifestations due to small numbers). There was significant seasonal variability in severity among these cases, with 38% of severe infections in January

Table 2

Streptococcus pyogenes emm types identified during the study period, Iceland, 1975–2012 (n=226 cases)

emm type	children	adults	total	p value
1	12	47	59	0.85
2	1	5	6	1.00
3	3	18	21	0.84
4	6	7	13	**0.04**
5	0	3	3	1.00
6	3	3	6	0.16
8	0	4	4	0.87
9	0	1	1	1.00
11	0	2	2	1.00
12	6	11	17	0.15
22	5	6	11	0.07
28	0	25	25	**<0.01**
43	0	1	1	1.00
58	0	1	1	1.00
75	0	8	8	1.00
77	0	3	3	1.00
81	0	3	3	1.00
84	0	1	1	1.00
85	0	1	1	1.00
87	1	5	6	1.00
89	1	24	25	0.06
94	1	4	5	1.00
102	0	1	1	1.00
110	0	2	2	1.00
118	0	1	1	1.00
Unknown	15	47	62	0.29
Total	**54**	**234**	**288**	

Overall, 226 (74%) of invasive isolates (dating from 1987 to 2012) were available for *emm* sequencing. The most common *emm* types were 1, 28 and 89 which comprised nearly half of all the cases. As shown, *emm* type 4 was significantly more common among children (p=0.04), whereas *emm* 28 was seen solely among adults (p=0.01).

Table 3

Clinical manifestations associated with invasive Group A streptococcal infection and *emm* types, Iceland, 1975–2012 (n=226 cases)

Syndrome	*emm* 1	*emm* 28	*emm* 89	*emm* 3	*emm* 12	Other *emm* types	*emm* type unknown	Total	Fatal	ICU and/or fatal
Arthritis	2	1	4	0	2	10	3	22 (7.6%)	0 (0%)	3 (13.6%)
Skin/soft tissue	5	9	10	6	8	27	30	95 (33.0%)	3 (3.2%)	7 (7.4%)
Other	10	3	5	5	2	11	11	47 (16.3%)	5 (10.6%)	11 (23.4%)
Respiratory tract infection	9	3	0	**3**	**1**	**3**	**4**	**23 (8.0%)**	**4 (17.4%)**	**13 (56.5%)**
Sepsis	22	8	2	6	3	17	11	69 (24.0%)	12 (17.4%)	24 (34.8%)
STSS	8	1	2	1	0	5	3	20 (6.9%)	6 (30.0%)	20 (100%)
NF	3	0	2	0	1	6	0	12 (4.2%)	4 (33.3%)	11 (91.7%)
Total	59	25	25	21	17	79	62	288	34	89
Fatal	12 (20.3%)	4 (16.0%)	0 (0%)	2 (9.5%)	2 (11.8%)	6 (7.6%)	8 (12.9%)			
ICU and/or fatal	27 (45.8%)	8 (32.0%)	6 (24.0%)	7 (33.3%)	3 (17.6%)	21 (26.6%)	11 (17.7%)			

ICU: intensive care unit; NF: necrotising fasciitis; STSS: streptococcal toxic shock syndrome.

The clinical presentation of *emm* 1, the most common *emm* type (25% of isolates), was in over 50% of cases sepsis, STSS and NF, while *emm* 28 (11% of isolates), 89 (11% of isolates) and 12 (8% of isolates) more often caused soft tissue infections.

to April compared with 16% of severe infections in other months (p<0.01). There was also significant seasonal variation in the severity of arthritis and non-focal infections: 32% were severe in January to April compared with 5% during the rest of the year (p<0.05). There were too few cases of non-severe NF and STSS to evaluate seasonal variations. Analyses of adults infected with *emm* type 1 showed that 75% of cases were severe in January to April compared with 37.5% in June to September, but this difference was not statistically significant (p=0.09, Fisher's exact test).

Discussion

We present a long-term population-based study of IGASI in Iceland, a setting where these infections are frequent compared with other Western developed nations. The study had a long observation period, and clinical as well as microbiological data were analysed.

The incidence of IGASI varies greatly by geographical location. Published studies from Europe show an incidence in northern European countries of 2.5 to 3.1 per 100.000 inhabitants per year in Finland, Denmark and Norway [6,7], which is within the range of the estimates presented here. During the last decade, the incidence rate in our study was 3.96 per 100.000 inhabitants per year, i.e. even higher than corresponding figures from other Nordic countries [6,7,13]. The results are also in accordance with other reports, which show an increase starting in the 1980s [21]. Currently, the global number of IGASI is estimated to be 663,000 new cases and 163,000 deaths each year, which is higher than that caused by measles [21,22].

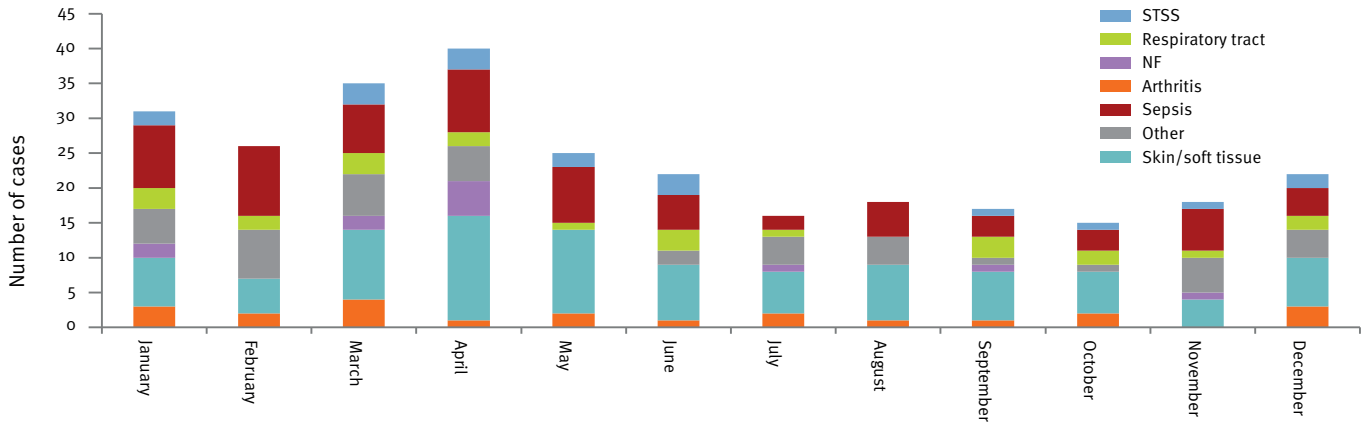
The reasons for the increased incidence are not yet entirely clear. In the first decade of the current study, more sensitive blood culture methods as well as improved access to these diagnostic tools may have played a role in improved detection. Such technical explanations are less plausible for the last 15 to 20 years of the study, when potential technical confounders have remained fairly unchanged. Another possible explanation is the periodic introduction of new, more virulent GAS strains, possibly carrying novel M proteins on their surface, for which there may be low herd immunity.

Our results show a higher age-specific incidence in the age groups under 10 and over 60 years-old, which is in agreement with other studies [2,13], probably reflecting the relative lack of sufficient immune responses at the extremes of age. Similarly, the most common clinical presentation is soft tissue infection followed by sepsis or bacteraemia without identified focus, which is in agreement with other studies [2,13,23]. Interestingly, there was a trend of more severe infections in the latter half of the study; the number of cases with NF and STSS almost doubled between 1995 to 2004 and 2005 to 2012, although the second interval was shorter. Other investigators have shown that 58–67% of individuals with IGASI have at least one underlying disease [11]. Malignancies, cardiovascular diseases, diabetes and alcoholism are most frequently implicated, and this is borne out by the current study. Chickenpox is also strongly associated with invasive bacterial super-infection with GAS [10]; only five children had chickenpox in our cohort, precluding meaningful calculations of the relative risk for GAS infections following chickenpox.

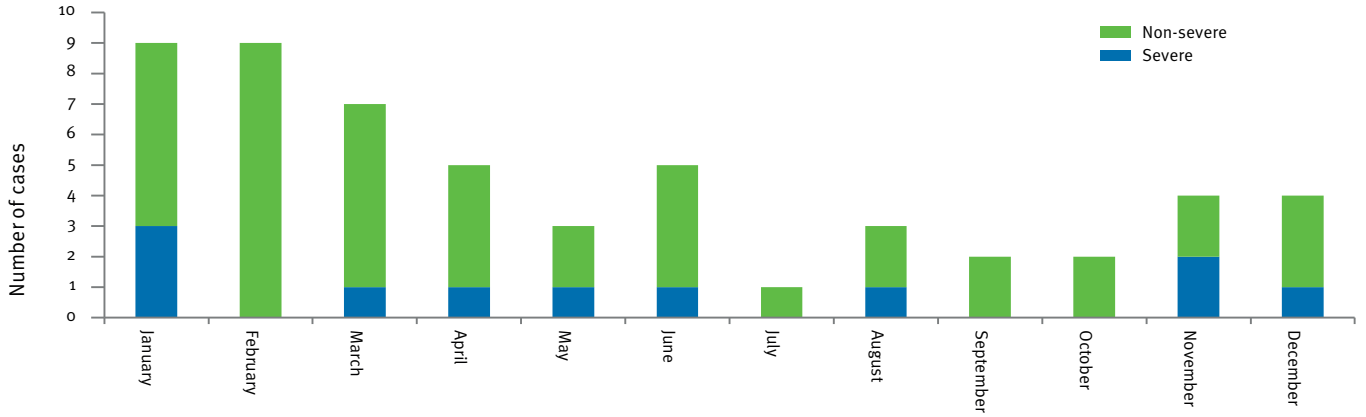
Figure 3

Seasonality, clinical manifestations and severity of invasive infections by Group A Streptococci in Iceland, 1975–2011 (n=285)

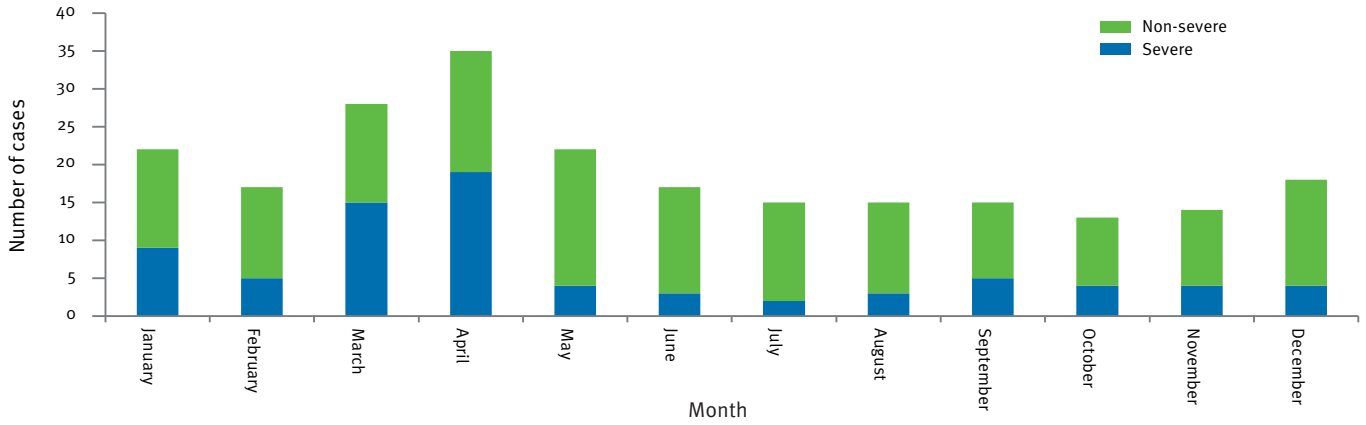
A. Clinical syndromes



B. Severe and non-severe cases in children (n=54)



C. Severe and non-severe cases in adults (n=231)



NF: necrotising fasciitis; STSS: streptococcal toxic shock syndrome.

A. The more aggressive cases (STSS, NF, sepsis) cluster in mid- to late winter.

B. The incidence reached a peak in January to February, but the severity was low in most cases, with no significant difference between winter and summer months (18% severe in summer vs 17% in winter).

C. The incidence peaked in April, with significant seasonal increase in severity, as defined as admission to intensive care and/or death.

The antibiotic treatment did not change during the study period. Penicillin is still the drug of choice in the treatment of streptococcal infections, with the addition of clindamycin in cases of STSS or NF and possibly intravenous immunoglobulin in select circumstances. No patient in this cohort received immunoglobulin.

In this study, the overall fatality was significantly higher among patients who developed STSS or NF. This is consistent with results from previous studies, including a study conducted in Sweden in 2002 to 2004 and a large European study in which 11 nations participated in the years 2003 and 2004 [11,13]. Almost half of all fatalities occurred within the first 48 hours of admission. These results are comparable to that of another study [24], emphasising the importance of early recognition and treatment.

A GAS clone carrying the M1 (*emm*1) surface protein was first identified in the mid-1980s and associated with resurgence of serious streptococcal infections [25]. The M protein, encoded by *emm*, is a fimbrial protein located on the cell surface and an important virulence factor with anti-phagocytic properties. On the other hand, the risk of severe IGASI may also be determined in part by the genetic susceptibility of the host [26,27]. Over a 25-year period, 25 different *emm* types were identified in Iceland. This *emm* distribution found in the current study was similar to that found in other Nordic countries [6,7]. In our cohort, *emm*4 was significantly more common among children, whereas *emm*28 was seen solely among adults. The reason for this discrepancy between children and adults is unclear but may be reflective of differences in specific immunity between age groups. In Europe, the most prevalent *emm* types in adults are, in descending order, *emm*1, *emm*28, *emm*3 and *emm*89; also in children, *emm*1 is the most common isolate, followed by *emm*12, *emm*4, *emm*3 and *emm*28 [14].

The study of associations between *emm* types and clinical manifestations is challenging due to the vast number of circulating *emm* types. Spread of otherwise uncommon *emm* types among disadvantaged populations has been well described [28,29]. There are indications that cellulitis may more often be caused by *emm*87, *emm*83 or *emm*81, whereas STSS and NF have been linked to *emm*1 and puerperal sepsis to *emm*28 [11,14]. Our study did suggest a significant association between infections caused by *emm*1 and severe disease, whereas patients with *emm*89 and 12 had less severe disease courses. The reasons why certain *emm* types are capable of causing more severe disease is unknown, but may be related to their associations with yet another virulence trait, such as production of SpeA, SpeZ and SpeJ exotoxins [30]. However, no correlation between clinical manifestations and a single superantigen gene in GAS has been found [11].

Seasonality of pharyngitis and invasive disease caused by GAS has been documented previously. Studies have shown, with remarkable congruence between countries, that the overall incidence of invasive infections varies by month [2,13]. We also observed significant seasonal variation, with the incidence among adults being highest in March and April. Underlying causes of seasonality are incompletely understood, but explanations such as preceding viral infections and seasonal changes in behavioural patterns, e.g. increased in-door activities during winter, have been proposed as potentially increasing the likelihood of transmission [2,13]. The similarity between countries may also be suggestive of an environmental factor occurring in several countries at the same time, such as decrease in sunlight and possibly simultaneous increased host susceptibility [31]. In support of this theory, we observed an association between severity of IGASI and seasonality, with a higher proportion of severe cases in January to April compared with the average in June to September. Another study from the United Kingdom [24] had also demonstrated a seasonal difference in case severity, with more severe infections diagnosed in January.

The seasonal variation that was noted here does correspond very closely to the traditional influenza season in Iceland [19]. In addition, concurrent infections with influenza and GAS have been reported, with an apparent increase in mortality when compared with influenza alone [32]. However, we tested for, but did not detect, an association between IGASI in Iceland and the influenza epidemic period. The number of cases occurring per season in this study may be insufficient to detect such an increase above the seasonal baseline. This is in contrast to a previous Canadian study which showed an association with Influenza B, which accounted for about 8% of the incidence of IGASI over any effect associated with modelled seasonal and long-term trends [33].

Seasonal variation in vitamin D levels has been proposed to affect immune function [34]. Indeed, previous work from Iceland has shown that vitamin D levels are lowest in February to March and highest in June to July [35]. Thus, it would be interesting to investigate further the impact vitamin D level has on severity of GAS infections. An alternate hypothesis of introduction of more virulent strains during the winter months was not borne out by our data, seeing as cases with *emm*1 type were more severe in winter than in summer. Likewise, the proportion of *emm*1 types among all adult disease isolates was comparable in winter (22%) and summer (15%).

In summary, GAS remains a major public health concern, with this pathogen showing great variability over time in incidence and clinical presentation. In this long-term, nationwide study, we show a seasonal increase in disease severity in patients with sepsis, respiratory infections or cellulitis caused by GAS during January

to April compared with other months. This pattern was also evident in patients with invasive arthritis and non-focal infections, suggesting that a generalised seasonal increase in host susceptibility may play a role in the pathogenesis of these common, severe infections.

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Conflict of interest

None declared.

Authors' contributions

Lovísa Björk Ólafsdóttir: reviewed clinical data, analysed the data and wrote the paper. Helga Erlendsdóttir: generated the microbiology registry and wrote the paper. Jose Melo-Cristino: performed *emm* typing of the isolates. Daniel M. Weinberger: performed statistical analysis and modelling and wrote the paper. Mario Ramirez: performed *emm* typing of the isolates and wrote the paper. Karl G. Kristinsson: generated the microbiology registry and wrote the paper. Magnús Gottfredsson: reviewed clinical and microbiology data, analysed the data and wrote the paper.

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Investigation of an association between onset of narcolepsy and vaccination with pandemic influenza vaccine, Ireland April 2009-December 2010

D O'Flanagan (darina.oflanagan@hse.ie)[1,2], A S Barret[1,2], M Foley[1], S Cotter[1], C Bonner[3], C Crowe[4], B Lynch[5], B Sweeney[6], H Johnson[7], B McCoy[8], E Purcell[4]

1. Health Service Executive, Health Protection Surveillance Centre, Dublin, Ireland
2. These authors contributed equally to this manuscript
3. Department of Health, Dublin, Ireland
4. Mater Private Hospital, Dublin, Ireland
5. Children's University Hospital Temple Street, Dublin, Ireland
6. Cork University Hospital, Cork, Ireland
7. Health Service Executive, Health Intelligence Unit, Dublin, Ireland
8. Our Lady's Children's Hospital, Crumlin, Dublin, Ireland

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In 2011, the Irish Medicines Board received reports of onset of narcolepsy following vaccination against influenza A(H1N1)pdm09 with Pandemrix. A national steering committee was convened to examine the association between narcolepsy and pandemic vaccination. We conducted a retrospective population-based cohort study. Narcolepsy cases with onset from 1 April 2009 to 31 December 2010 were identified through active case finding. Narcolepsy history was gathered from medical records. Pandemic vaccination status was obtained from vaccination databases. Two independent experts classified cases using the Brighton case definition. Date of onset was defined as date of first healthcare contact for narcolepsy symptoms. Incidence of narcolepsy in vaccinated and non-vaccinated individuals was compared. Of 32 narcolepsy cases identified, 28 occurred in children/adolescents and for 24 first healthcare contact was between April 2009 and December 2010. Narcolepsy incidence was 5.7 (95% confidence interval (CI): 3.4–8.9) per 100,000 children/adolescents vaccinated with Pandemrix and 0.4 (95% CI: 0.1–1.0) per 100,000 unvaccinated children/adolescents (relative risk: 13.9; absolute attributable risk: 5.3 cases per 100,000 vaccinated children/adolescents). This study confirms the crude association between Pandemrix vaccination and narcolepsy as observed in Finland and Sweden. The vaccine is no longer in use in Ireland. Further studies are needed to explore the immunogenetic mechanism of narcolepsy.

Introduction

Narcolepsy is a sleep disorder characterised by excessive daytime sleepiness (EDS) and is often associated with cataplexy (episodic muscle weakness) triggered by emotion such as laughter or anger. Nocturnal sleep is usually fragmented and may be associated with sleep paralysis and hypnagogic hallucinations. Other symptoms may include weight gain and obesity, deterioration in school performance and emotional lability [1].

The precise aetiology of narcolepsy is unknown but it is generally considered to be triggered by a combination of genetic and environmental factors. An important predisposing genetic factor is a specific human leukocyte antigen (HLA), the HLA DQB1*0602. Patients with narcolepsy-cataplexy carry the allele DQB1*0602 in 85–95% of cases, compared with about 30% of the general population [2]. Narcolepsy results from a decrease in levels of the neuropeptides hypocretin-1 and -2. This loss is caused by destruction of the hypocretin-producing cells in the hypothalamus region in the brain. Narcolepsy diagnosis can be confirmed by hypocretin-1 (orexin-A) measurement in the cerebrospinal fluid (CSF). This test has a high sensitivity and specificity in patients with typical cataplexy [3]. In patients without cataplexy, the test of reference is the multiple sleep latency test (MSLT).

Possible environmental risk factors for narcolepsy include streptococcal infection and viral infections including influenza [4-6]. The prevalence of narcolepsy with cataplexy is estimated at 25–50 per 100,000 population in western industrialised countries. The incidence has been estimated at 0.74 cases per 100,000 person-years for narcolepsy with cataplexy and 1.37 for narcolepsy without cataplexy [2,7]. In most cases age at onset of symptoms is between 15 and 40 years. However, the disease often goes unrecognised and undiagnosed for many years, ranging from 1–61 years in a United Kingdom (UK) study [8].

In August 2010, the Swedish Medical Product Agency and the Finnish National Institute for Health and Welfare (THL) reported cases of narcolepsy as possible adverse events following vaccination against influenza A(H1N1) pdm09 with Pandemrix [9,10]. By the end of March 2011, the Irish Medicines Board (the Irish pharmacovigilance authority) had also received reports of two confirmed cases of narcolepsy following vaccination with Pandemrix. In Ireland, two vaccine brands were used during the 2009-2010 pandemic influenza vaccination campaign: Pandemrix (GlaxoSmithKline) and Celvapan (Baxter). Celvapan was a whole-cell killed vaccine produced on a Vero cell line and did not contain any adjuvant. Pandemrix was an inactivated split influenza virus vaccine produced in eggs. It contained the adjuvant AS03. Overall 88% of those receiving at least one dose of pandemic vaccine were vaccinated with Pandemrix and 12% with Celvapan. Both vaccines were used in the early phase of the pandemic vaccination programme when the at risk groups were prioritised to receive vaccine[11]. In the later phases, Pandemrix was mainly used when the vaccine was offered to children and adults outside of the main risk groups.

The objective of this study was to investigate if the risk of onset of narcolepsy was increased in the Irish population who received pandemic vaccine in comparison with those not receiving pandemic vaccine. The results of the investigation were presented in the final report of the National Narcolepsy Study Steering Committee in April 2012 [11]. The results reported here have been updated using the newly available 2011 census data (as opposed to 2006 census data in the final Department of Health report).

Methods

We conducted a retrospective population-based cohort study in order to investigate the association of narcolepsy with the pandemic vaccination in Ireland by comparing the incidence of narcolepsy in vaccinated and non-vaccinated individuals from 1 April 2009 to 31 December 2010.

Study population

Based on the preliminary signal of an increase in the incidence of narcolepsy in children and adolescents, three cohorts were defined: children aged 0–4 years, children/adolescents aged 5–19 years, and adults aged 20 years and over. However, no narcolepsy cases were reported in children aged 0–4 years in Ireland. Therefore the analysis was performed only for the two other cohorts, i.e. children/adolescents aged 5–19 years and adults. The 2011 census data by single year of age was used to estimate the number of the Irish population in each age group. We used similar age groups to studies conducted in other countries e.g. 4–19 year-olds in Finland [12], 4–18 year-olds in the United Kingdom [13], 4–19 year-olds in Norway [14] and 5–19 years in the Vaccine Adverse Event Surveillance & Communication (VAESCO) study [15,16].

Exposure to pandemic vaccination

In Ireland, the Health Service Executive (HSE) provided the pandemic vaccine to the population in several phases between November 2009 and March 2010. Those at highest risk of influenza and its complications received the vaccine in the early stages i.e. from 12 October 2009. Vaccination of healthy children aged less than 18 years started from 30 November 2009. Information on vaccination was collected in one of two databases, depending on where vaccination was administered: vaccination performed in general practitioner (GP) clinics were registered in the Primary care reimbursement service (PCRS) database and those in HSE mass vaccination clinics in the Pandemic data management system (PDMS) database. For each cohort, we extracted from the databases the number of individuals vaccinated with Pandemrix by week of vaccination. The number of unvaccinated individuals was computed by subtracting the number of individuals vaccinated with any pandemic vaccine brand from the total number of individuals reported in the 2011 census.

Case finding and ascertainment

Narcolepsy cases were identified through active case finding from April 2011 to October 2011 by contacting all sleep clinics, neurologists, paediatricians, GPs, psychiatrists, psychologists and public health nurses in Ireland. It was emphasised to investigating

Table 1

Brighton collaboration case definition for narcolepsy [16,17][a]

Level	Criteria
Level 1	Excessive daytime sleepiness AND/OR suspected cataplexy AND CSF hypocretin-1 deficiency
Level 2	Excessive daytime sleepiness AND Definite cataplexy AND Level 1 or 2 MLST abnormalities (mean sleep latency ‹8 minutes for adults and ‹12 minutes for children ‹16 years AND/OR at least 2 sleep-onset REM periods)
Level 3	Excessive daytime sleepiness AND Level 1 MLST abnormalities (mean sleep latency ‹8 minutes for adults and ‹12 minutes for children ‹16 years AND at least 2 sleep-onset REM period)
All levels	In the absence of other mimicking disorders

CSF: cerebrospinal fluid; MLST: multiple sleep latency test.

[a] A Brighton Collaboration working group created a case classification to support the Vaccine Adverse Event Surveillance & Communication (VAESCO) study. Although the final document had not been published the above draft criteria were utilised in the Irish study.

neurologists and sleep clinics that cases should be reported regardless of exposure history. The various professional groups were individually contacted by mail and follow up was made by telephone contact with sleep clinics to confirm if no cases were observed. Two experts (one adult and one paediatric neurologist) reviewed the clinical history of narcolepsy cases in order to confirm the diagnosis and classify them using the internationally agreed Brighton Collaboration case definition for narcolepsy [17] (Table 1). The reviewers were blinded to the vaccination status of the cases. Neither of the independent experts were involved with the diagnosis or management of the cases included in the study. Cases were included in the study if (i) their date of first symptom of narcolepsy recorded in medical files occurred after 1 April 2009 and before 31 December 2010, (ii) cases or guardians gave oral informed consent, (iii) they were classified as level 1,2 or 3 as per the Brighton case definition. Prevalent cases with onset prior to April 2009 were excluded.

Data collection

Medical and narcolepsy history was collected from the medical records and clinical charts. Data included demographic information, details on history of narcolepsy, human leucocyte antigen (HLA) type, hypocretin-1 level and information on pandemic vaccination. For each narcolepsy case, we cross-checked the pandemic vaccination status, date of vaccination and vaccine brand in the PCRS and PDMS databases. CSF hypocretin-1 level values less than 50 pg/ml were considered as undetectable and highly suggestive of narcolepsy/cataplexy.

Estimation of the date of onset of narcolepsy

Because the date of onset of narcolepsy is often uncertain and date of diagnosis may be delayed, we used the date of first contact with healthcare for narcolepsy symptoms as retrieved from GP notes and clinical records to estimate the onset of narcolepsy. This date was considered as the most reliable and objective to estimate the onset of narcolepsy. Other dates were used in sensitivity analyses. The following dates were retrieved:

- Date of onset of first symptom of narcolepsy recalled by the patient or their parents (first documented history of symptoms such as excessive fatigue, sleep attacks, symptoms suggestive of cataplexy, etc.). When the exact date of symptom onset was unknown, it was approximated to the 15 of the reported month.
- Date of referral to a specialist for a sleep test.
- Date of MSLT which concluded the diagnosis of narcolepsy.

In the primary analysis, an incident case of narcolepsy was defined as having had a first contact with healthcare for narcolepsy symptoms during the study period. In the sensitivity analyses, we included cases with (i) date of onset, (ii) date of referral, (iii) date of MLST within the study period (right-censored cases). In all analyses, an incident exposed case was defined as having received one or more dose of Pandemrix before the recalled date of first symptom of narcolepsy. Cases who had received the first dose of Pandemrix after the recalled symptom onset were considered as unvaccinated. The only narcolepsy case who had received Celvapan had the first contact with healthcare after 31 December 2010 and was therefore not included in the analysis of the risk of narcolepsy.

In Ireland, the suspected risk of narcolepsy associated with pandemic vaccination was not communicated to healthcare professionals and to the public before March 2011. Therefore we considered that individuals who consulted, were referred for a sleep test or diagnosed before this date were not associated with an increase in the awareness of professionals. As a proxy for the effect of media attention in the population, we also looked at trends in searches for the keyword 'narcolepsy' in Google Trends.

Statistical analysis

The primary follow-up time was defined from 1 April 2009 to 31 December 2010. Two further study periods were defined in the sensitivity analyses:

- Period 2 from 1 April 2009 to 15 August 2010, before the increased media attention that occurred in Sweden and Finland, respectively at the end of August 2010;
- Period 3 from 01 October 2009, after the pandemic vaccine became available in Ireland, up to 31 December 2010.

Cases were described according to their demographic and clinical characteristics. Bivariate analysis was conducted to compare the characteristics of Pandemrix-vaccinated cases and unvaccinated cases. Differences were tested using Fisher exact test for categorical variables and Mann–Whitney test for quantitative variables.

The incidence of narcolepsy was calculated by dividing the number of cases by the follow-up time. The risk time (person-years) in Pandemrix-vaccinated individuals was calculated from the week of first vaccination until the end of the study period. The risk time in unvaccinated individuals was calculated from 1 April 2009 until the end of the study period for unvaccinated individuals, added to the time from 1 April 2009 to the week preceding vaccination for Pandemrix-vaccinated individuals.

In the sensitivity analyses of the risk of narcolepsy, we also used more specific case definitions: either cases with cataplexy or cases classified as level 1 in the Brighton case definition.

The relative risk (RR) was calculated as the ratio of the incidence rates for those vaccinated and unvaccinated,

Table 2

Characteristics of narcolepsy cases by vaccination status, April 2009-December 2010, Ireland (n=32)

Characteristics	All cases (N=32) n (%)	Vaccinated cases (N=24)[a] n (%)	Unvaccinated cases (N=7)[a] n (%)
Age at disease onset			
5-9	10 (31)	9 (38)	1 (14)
10-14	14 (44)	11 (46)	3 (43)
15-19	4 (13)	2 (8)	2 (29)
≥20	4 (13)	2 (8)	1 (14)
Sex			
Male	10 (31)	8 (33)	2 (29)
Female	22 (69)	16 (67)	5 (71)
Brighton case definition			
Level 1	15 (47)	14 (58)	1 (14)
Level 2	12 (37)	8 (33)	3 (43)
Level 3	5 (16)	2 (8)	3 (43)
Presence of cataplexy			
Yes	24 (75)	19 (79)	4 (57)
Time in days between onset of EDS and cataplexy			
No. of cases with information	19	16	2
Median	62	62	-
Minimum-maximum	0-365	0-365	76-243
Q1-Q3	31-165	31-142	-
HLA typing			
No. of test performed	23	20	3
No. of cases with DQB1*0602	23	20	3
Hypocretin test			
No. of tests performed	19	17	2
No. with hypocretin-1 level values <50 pg/ml	18	17	1

EDS: excessive daytime sleepiness; HLA: human leukocyte antigen; No: number; Q: quartile.

[a] One case vaccinated with Celvapan excluded in this analysis.

and the absolute attributable risk (AAR) was calculated as the difference in the incidence rates. We performed univariate Poisson regression using aggregated data in Stata version 11 to calculate the 95% confidence intervals (CI) around the RR. StatsDirect version 2.7.9 was used to compute the 95% CI around the AAR.

Ethical approval

The study protocol was reviewed and approved by the Faculty of Public Health Medicine Ethics Committee, Royal College of Physicians Ireland.

Results

The study cohorts comprised 906,280 children/adolescents and 3,325,643 adults.

Pandemic vaccination data

According to the PCRS and PDMS databases, 946,795 individuals received a first dose of Pandemrix between 12 October 2009 and 31 December 2010, a total of 88% of all first doses applied of pandemic vaccine. Pandemrix vaccine uptake was 20.8% across all age groups, 40.3% in those aged 0–4 years, 37.8% in those aged 5–19 years, and 14.3% in those aged 20 years and over.

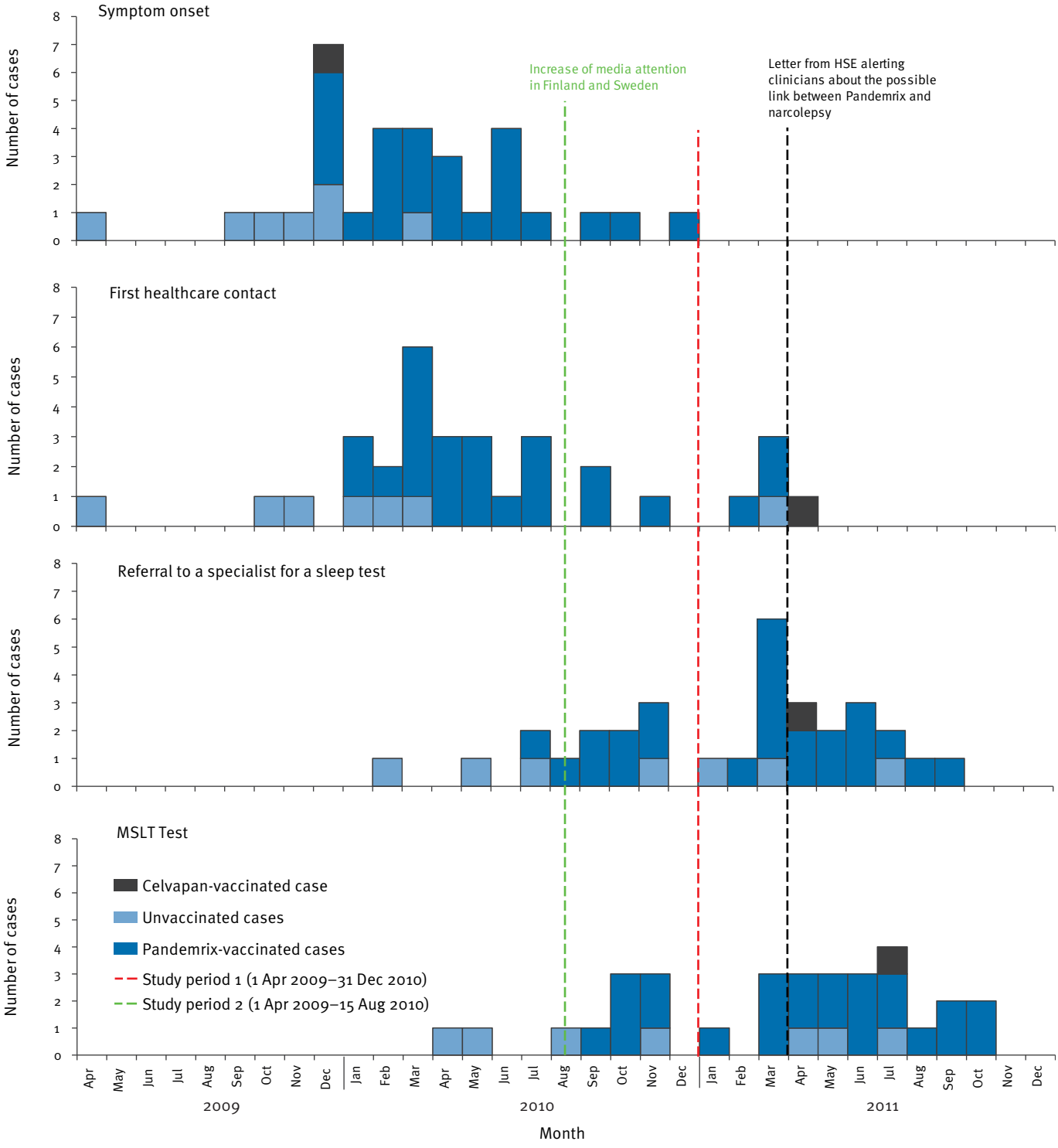
Description of narcolepsy cases

The active case finding identified 63 cases of narcolepsy. Of these, 25 cases had an onset of symptom prior to April 2009, one case did not agree to participate in the study and five cases did not meet the case definition. Thirty-two cases met the inclusion criteria and were included in the study.

Cases were predominantly reported from paediatric neurologists in Dublin and an individual specialist in sleep disorders with a particular interest in narcolepsy. Cases were referred to these centres from all over Ireland and there was no marked geographical clustering. All sleep clinics responded after telephone contact. There was a 100% concordance between the two evaluators when classifying the cases according to the Brighton Collaboration case definition. Based on the age at symptom onset, 28 of 32 cases were less than 20 years old and four cases were adults. Cataplexy occurred in all four adults and 20 of the 28 children/adolescents. HLA typing was performed in 23 cases and all of them presented the HLA DQB1*0602 allele. Table 2 describes the characteristics of the 32 narcolepsy cases by vaccination status.

Figure 1

Distribution of narcolepsy cases according to different estimated onset dates and by vaccination status, April 2009–December 2011, Ireland (n=32)



HSE: Health Service Executive; MLST: multiple sleep latency test.

Note: When the exact date of symptom onset was unknown, it was approximated to the 15 of the reported month.

Table 3

Delay between vaccination against influenza A(H1N1)pdm09 with Pandemrix and symptom onset in vaccinated narcolepsy cases, Ireland, April 2009-December 2010 (n=24)

Delay between vaccination and symptom onset	Number of cases	Cumulative proportion (%)
0–6 days (<1 week after vaccination)	1	4.2%
7–27 days (1–3 weeks after vaccination)	2	12.5%
28–55 days (4–7 weeks after vaccination)	5	33.3%
56–83 days (8 to <12 weeks after vaccination)	6	58.3%
3 months	3	70.8%
4 months	2	79.2%
5 months	1	83.3%
6 months	1	87.5%
7 months	1	91.7%
8 months	1	95.8%
9 months	0	95.8%
10 months	0	95.8%
11 months	0	95.8%
12 months	1	100.0%

The distribution of cases over time using different proxy dates for estimating the date of onset is presented in figure 1, as well as the sensitivity analyses. Twenty-seven of 32 cases had their first contact with healthcare for narcolepsy symptoms within the primary study period.

The median delays between the recalled symptom onset and the first healthcare contact, the first referral to a specialist for a sleep test, and the date of MLST were 1.8 months (range 0 to 15.6), 12.7 months (range 3.0 to 17.5) and 12.7 months (range 3.9 to 19.3), respectively.

Of 32 cases, 24 had been vaccinated with Pandemrix before the recalled onset of the first narcolepsy symptom; five cases had received Pandemrix after the first symptom onset; one case had received Celvapan; two cases had never received any pandemic vaccine. All but one of the vaccinated cases had received one dose of vaccine. There was no association with any particular batch number and although nine cases had received a particular batch, it was the most common batch number reported in the PCRS and PDMS databases. For 24 vaccinated cases, the median delay between the vaccination and the first symptom of narcolepsy was 2.2 months (range 6 days to 12.8 months). The distribution of the delay between vaccination and symptom onset is shown in table 3. The median delay between the vaccination and the first healthcare contact for narcolepsy symptom was 4.1 months (range 1.8 months to 16.0 months). Of 24 cases vaccinated prior to onset of symptoms, 20 were HLA-typed and all 20 cases presented the DQB1*0602 allele. The median age for these 20 was 11 years (range 5 to 17), seven were males and 13 females.

Comparison of Pandemrix-vaccinated and unvaccinated cases

Vaccinated cases (n=24) and unvaccinated cases (n=7) did not differ significantly in the age distribution (Fisher exact test, p=0.36) and male/female ratio (Fisher exact test, p=1.0). There was a significant difference in the levels of the Brighton Collaboration case definition, with vaccinated cases being more frequently classified as level 1 than unvaccinated cases (58% vs. 14%, Fisher exact test p=0.03). Cataplexy was more frequent in vaccinated than unvaccinated cases although the difference was not statistically different (79% vs. 57%, Fisher exact test p=0.33).

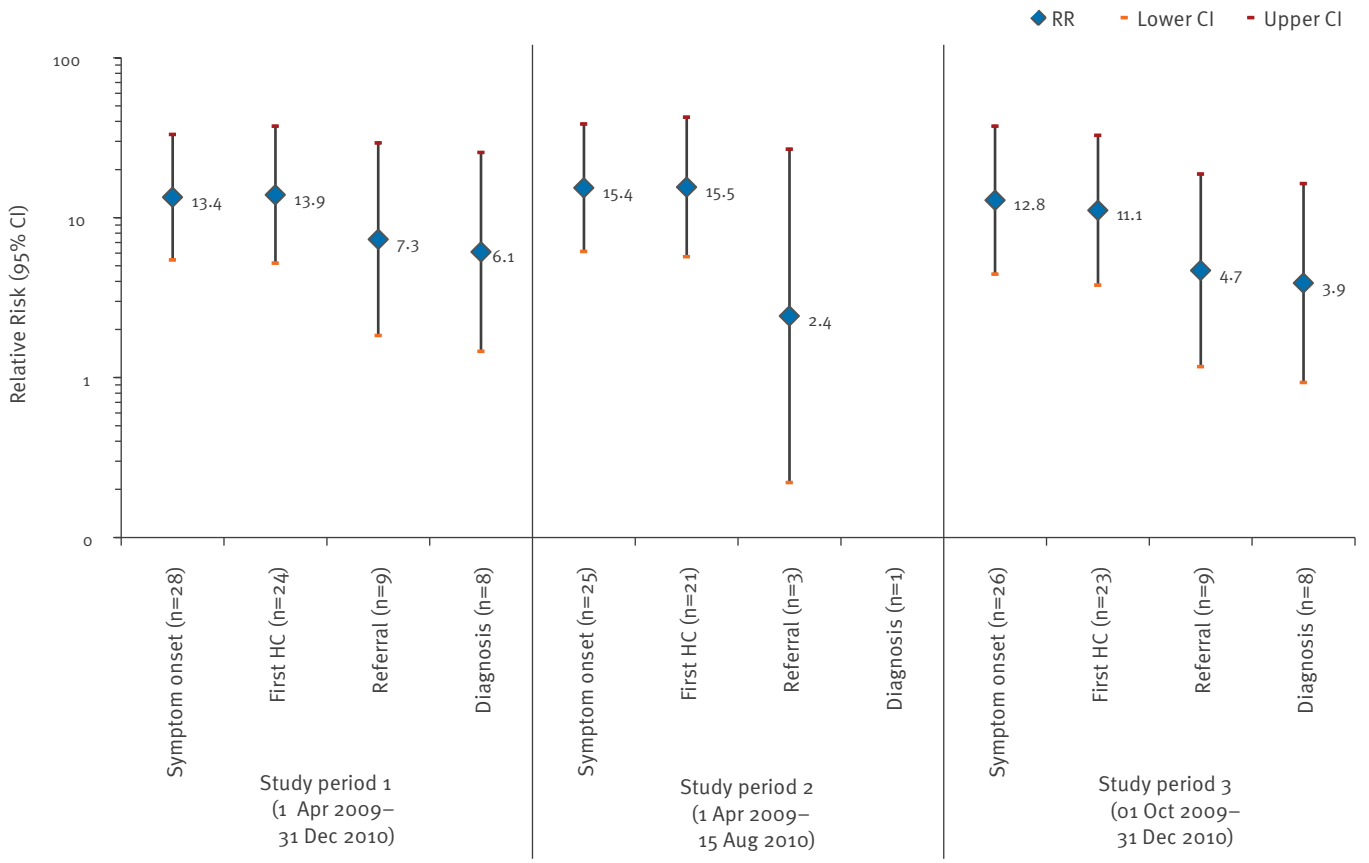
Risk of narcolepsy in children/adolescents

The primary analysis included 24 narcolepsy cases with a first contact with healthcare because of narcolepsy symptom within the primary study period. Nineteen cases were vaccinated and five were unvaccinated. The incidence of narcolepsy during the primary follow-up time was 5.7 (95% CI: 3.4–8.9) per 100,000 person years in the vaccinated and 0.4 (95% CI: 0.1–1.0 per 100,000 person years in the unvaccinated individuals. The RR was 13.9 (95% CI: 5.2–37.2) and the AAR associated with the vaccine was 5.3 narcolepsy cases per 100,000 vaccinated children/adolescents (95% CI: 3.8–6.8). In sensitivity analyses using different periods and onset dates, the RR remained consistently greater than one. The lower limit of the 95% CI was greater than one in eight analyses and the lower limit below one in two analyses (Figure 2).

Considering only those cases presenting with cataplexy (14 vaccinated, 3 unvaccinated cases), the RR was 17.1 (95% CI: 4.9–59.4) in the primary analysis. Considering cases classified as level 1 in the Brighton case definition (13 vaccinated cases, 1 unvaccinated case), the RR was 47.5 (95% CI: 6.2–363.5) in the primary analysis.

Figure 2

Sensitivity analysis for relative risk of narcolepsy in vaccinated (n=22) compared with unvaccinated children and adolescents (n=6) aged 5–19 years using different index dates and study periods, Ireland April 2009–December 2010



CI: confidence interval; HC: healthcare contact; RR: relative risk.

Risk of narcolepsy in adults

The analysis included three adult narcolepsy cases with a first contact with healthcare because of narcolepsy symptom within the primary study period. Two cases were vaccinated with Pandemrix and one case was unvaccinated. The incidence of narcolepsy during the primary follow-up time was 0.39 (95% CI: 0.05–1.42) per 100,000 person years in the vaccinated adults and 0.02 (95% CI: 0.0005-0.11) per 100,000 person-years in the unvaccinated adults. The RR was 20.4 (95% CI: 1.8–225.0) and the AAR associated with the vaccine was 0.37 narcolepsy cases per 100,000 vaccinated adults. Given the small number of cases in adults, the sensitivity analysis using different index dates or study periods could not be done. One adult case vaccinated with Celvapan was not included in the analysis.

Media attention

The trends in searches for the keyword 'narcolepsy' in Google suggested that concerns about narcolepsy in the Irish population started to rise in September 2011 as opposed to August 2010 in both Finland and Sweden (Figure 3).

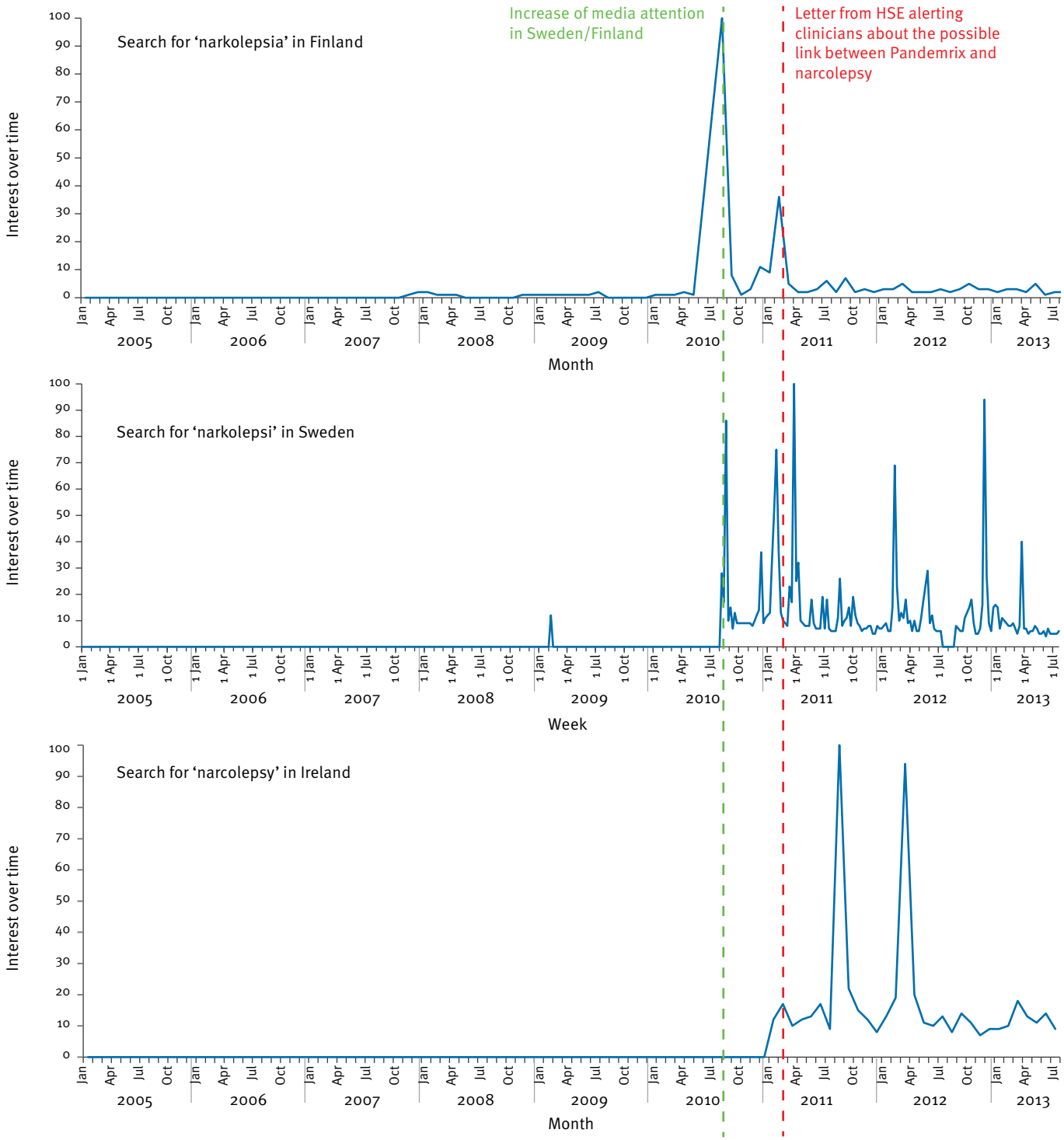
Discussion

In Ireland, there is limited information on the epidemiology of narcolepsy. An Irish study conducted in 2009 estimated the prevalence of narcolepsy at five per 100,000 population. Most reported patients belonged to the age group of 13–19 year-olds. The estimate of prevalence based on this study was substantially less than the estimated prevalence rate reported in other western countries [7]. Although the authors did not rule out a low prevalence rate due to Irish unique ethnicity, they concluded that narcolepsy prevalence rates were largely underestimated possibly because of the misinterpretation of reported symptoms of EDS, the unclear care management of patients with suspected narcolepsy, the possibility of false negative sleep test results and the financial and logistical constraints of hypocretin-1 testing [18].

It is unlikely that this low prevalence is related to genetic non-susceptibility. A study of frequencies of HLA class I and II alleles and haplotypes of 250 Irish unrelated bone marrow donors found that the HLA DQB1*0602 was present in 35% of them [19]. As there is no register for narcolepsy in Ireland it is impossible

Figure 3

Google Trends searches for the keyword 'narcolepsy' in Finland, Ireland and Sweden, January 2005–August 2013



HSE: Health Service Executive.

Source: Google Trends - www.google.com/trends

Note from Google Trends: For queries that have low popularity, the breakdown is given by month, not week. In these cases, the monthly breakdown usually creates a smoother curve on the graph, making it easier to analyse any changes.

to comment on the background incidence rates of narcolepsy prior to 2009.

Our study found a significant, 13.9-fold higher, risk of narcolepsy in children/adolescents vaccinated in Ireland with Pandemrix compared with unvaccinated children/adolescents. The absolute number of narcolepsy cases attributable to Pandemrix vaccination was five per 100,000 vaccinated children/adolescents or one in 19,000 vaccinated (95% CI: 1 in 15,000–1 in 26,000). These findings are remarkably similar to the results found in the retrospective population-based cohort study conducted in Finland in 2011 [12]. This study showed a 12.7 fold higher risk of developing narcolepsy in children/adolescents vaccinated with Pandemrix as compared with unvaccinated children/adolescents. The vaccine attributable risk of developing narcolepsy was one in 16,000 vaccinated 4–19 year-olds (95% CI: 1 in 13,000–1 in 21,000).

These vaccine attributable risks in Finland and Ireland are much higher than that recently reported in England where it was estimated as between one in 57,500 and one in 52,000 doses [13]. Miller et al. postulated that the lower attributable risk found in the UK may be related to a lower genetic susceptibility in England or because proportionately more vaccine was given in Finland and Ireland to adolescents, in whom incidence of narcolepsy is highest. Another possibility is that there remains underascertainment of narcolepsy cases in countries where there was neither active case finding nor widespread media coverage. It is likely that active case finding in Ireland led to improved detection and appropriate referral for diagnosis. Wijnans et al. reported on the incidence of narcolepsy in Europe before, during and after the influenza A(H1N1)pdm09 pandemic and vaccination campaigns. The study used large linked databases in six countries in Europe [15]. Increases in incidence rates after the start of pandemic vaccination were detected in 5–19 year-olds in Denmark, Finland and Sweden but not in Italy, Netherlands, and the UK. Case verification was undertaken using data in the Dutch database and resulted in exclusion of 50% of initially identified cases in the Netherlands. The failure to detect the subsequently identified risk in the UK together with the verification problems identified in the Netherlands, indicates limitations to the sole use of large databases for the purpose of ruling out vaccine associated safety signals.

The primary study period in this study was chosen to include all cases with a possible exposure to both pandemic influenza infection and vaccination. However, when considering different index dates in the sensitivity analysis, excluding the cases that sought healthcare or were referred or had a MLST test after 31 December 2010, the RR remained consistently and significantly greater than one. We obtained similar results when restricting the study period to starting October 2009, when the pandemic vaccine had been made available to the Irish population.

The health-seeking behaviour, referral for a sleep test or numbers of cases diagnosed did not seem to have changed in Ireland following the increase of media attention in Finland. Looking at the earliest medical report dates of the cases of narcolepsy, we neither found an increase in healthcare-seeking behaviour, nor referral to specialists, nor diagnosis after August 2010. Whereas, a substantial number of cases were referred and diagnosed from March 2011 onwards. This coincided with communication to GPs in March 2011 by the Director of Health Protection, followed by communication to sleep clinics and paediatric and adult neurologists in April by the study investigators. As stated above, Google searches in Ireland suggest that concerns about narcolepsy in the Irish population started to rise in September 2011 as opposed to August 2010 in Finland and Sweden.

A source of potential bias in case selection may have been introduced by the use of active case finding in Ireland. Both GPs and hospital clinicians were made aware of the reason for the study. Clinicians were requested to investigate vaccinated and unvaccinated cases in a similar fashion. Although the case finding was performed irrespective of the cases' exposure status, it may be possible that vaccinated cases were more likely to have been identified. The RR based on the first contact with healthcare would become non-significant only if there were still an additional 36 unvaccinated cases to be diagnosed in the 5–19 year-olds in Ireland (RR: 1.70, 95% CI: 0.98-2.92). The RR point estimate would approach one only if there were still an additional 60 unvaccinated cases to be diagnosed in the 5–19 year-olds (RR:1.07, 95% CI: 0.6–1.8).

It is possible that GPs were more likely to refer vaccinated cases who had more subtle symptoms than those unvaccinated with subtle symptoms to specialists. However, we believe that GPs would refer those with severe symptoms of narcolepsy and cataplexy regardless of exposure history. If we include only children/adolescents with severe symptoms such as cataplexy, the increased risk is still observed. Therefore, we think it is unlikely that the active case finding can account for the strong association with Pandemrix described in this study. In addition there is an ethical issue to be considered in investigation of rare potential side effects of vaccines or other medications. It is essential that affected children and adolescents are detected and referred for appropriate diagnosis and treatment. This creates a dilemma as it is also important to attribute causality with as little introduction of bias as possible. This dilemma is perhaps more marked with a disease that has an insidious onset as has narcolepsy. It is possible that excessive consideration of diagnostic bias accounts for the results of the primary analysis of the VAESCO study. In this study data from five European countries without a narcolepsy signal and with varying vaccination coverage in children and adolescents were pooled and it failed to find an association between the use of Pandemrix and onset of narcolepsy [16]. The

same study confirmed the association in the 'signalling' countries Finland and Sweden. In the analysis the referral to MSLT was required to have happened before the initial media reports from Finland. This approach would have excluded all but one of the Irish cases from the analysis.

We identified very few narcolepsy cases in adults through our case finding and this limited the analysis of the risk of narcolepsy associated with pandemic vaccination. Although the RR point estimate for Pandemrix was relatively high, the wide CI around the estimate impedes drawing any conclusion for adults. A significant association between pandemic vaccination and narcolepsy has been reported in adults in France [20]. A more recent study published from the Swedish Medical products agency reports an increased risk in those aged 21–30 years [21]. In May 2013, the THL in Finland also reported an increased risk of narcolepsy in young adults, although the risk was lower than that previously reported in children and adolescents [22].

It is interesting that the more recent data from Sweden has also noted an increase of diagnosed cases of narcolepsy in unvaccinated children and adolescents looking at data from October 2009 up to December 2011. Recent Norwegian studies have shown a decline in the incidence in vaccinated children aged 4–19 years in the second year after vaccination, falling to levels not significantly different from unvaccinated children during the same period [14]. Further work will be required to examine if that can be replicated in Ireland and elsewhere.

Because of the small number of cases, we had a very low power for testing the difference of characteristics between vaccinated and unvaccinated cases. Vaccinated cases were more likely to be classified as level 1 of the Brighton case definition and to present with cataplexy although this latter finding was not statistically significant. The delay between EDS onset and cataplexy onset was also shorter in vaccinated cases compared with unvaccinated cases, although not statistically significant. These results might suggest a clearer clinical picture of narcolepsy in vaccinated cases and a possible quicker development of symptoms as compared with the classical presentation.

In this study, we could not adjust the analysis for possible confounding factors such as previous infections or other vaccinations. In Ireland, children and adolescents were the group most affected during the 2009-2010 influenza A(H1N1) pandemic. In the 5–14 year-olds, the influenza-like illness incidence rate peaked in week 43 (starting 25 October 2009), i.e. one week before the pandemic influenza vaccination campaign was officially launched. The combination of pandemic influenza infection and vaccination might have initiated the development of narcolepsy. However, a serological study conducted in Finland does not support this hypothesis. In this study, only two of 45 Pandemrix-vaccinated narcolepsy patients showed specific antibody response against the NS1 protein from the influenza A(H1N1)pdm09 virus [23]. Further studies are needed to explore other triggering factors and possible interactions.

It is noteworthy that a high number of cases in Ireland had the HLA allele DQB1*0602, all 23 tested were positive for this allele. The immunogenetic mechanism of narcolepsy and how Pandemrix vaccination contributed to its development need to be further studied and understood. While a number of European countries have now reported an increase in cases of narcolepsy associated with use of Pandemrix vaccine as yet there has been no report of an increase with the similar vaccine also produced by Glaxo Smith Kline, Arepanrix. Arepanrix also contains the adjuvant AS03 and was used extensively in Canada. This could imply that it is not the adjuvant per se that accounts for the association with narcolepsy observed with the use of Pandemrix. Interestingly the European Centre for Disease Prevention and Control has pointed to differences in the manufacturing process for these two vaccines [24]. Further study of these differences may help to explain the pathogenic processes involved in the triggering of narcolepsy.

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Conflict of interest

None declared.

Authors' contributions

All authors contributed to the interpretation of the study, the revision of the draft manuscript and approved the final version. Darina O'Flanagan chaired the steering committee for the study, led the drafting of the manuscript and the original methodological design of the study. Anne-Sophie Barret conducted the data analysis and participated in drafting the manuscript. Margaret Foley conducted the collection and collation of the study data. Suzanne Cotter participated in the steering committee and provided the vaccine denominator data. Colette Bonner participated in the steering committee and agreed report and final paper. Catherine Crowe participated in the steering committee and provided much of the clinical data. The majority of the sleep studies were performed in her laboratory in conjunction with Dr Elaine Purcell. Bryan Lynch participated in the steering committee and in case identification, also in investigation and ongoing care of the majority of the children in the study. Brian Sweeney contributed the adult neurological perspective to the design and execution of the study. He also independently verified the cases of narcolepsy by applying the recommended criteria to each case. Howard Johnson contributed to the study design, data interpretation and drafting of the manuscript.

Blathnaid McCoy conducted a blinded review of the clinical details of all cases to confirm diagnosis and classify according to established criteria. Elaine Purcell participated in the steering committee and provided much of the clinical data. The majority of the sleep studies were performed in her laboratory in conjunction with Dr Catherine Crowe.

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Emergence of *Escherichia coli* encoding Shiga toxin 2f in human Shiga toxin-producing *E. coli* (STEC) infections in the Netherlands, January 2008 to December 2011

I Friesema (ingrid.friesema@rivm.nl)[1], K van der Zwaluw[1], T Schuurman[2], M Kooistra-Smid[3], E Franz[1], Y van Duynhoven[1], W van Pelt[1]

1. Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
2. Medical Microbiology, section Virology, University Medical Centre Groningen, Groningen, the Netherlands
3. Department of Research and Development, Laboratory for Infectious Diseases, Groningen, the Netherlands

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The Shiga toxins of Shiga toxin-producing *Escherichia coli* (STEC) can be divided into Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) with several sub-variants. Variant *Stx*$_{2f}$ is one of the latest described, but has been rarely associated with symptomatic human infections. In the enhanced STEC surveillance in the Netherlands, 198 STEC O157 cases and 351 STEC non-O157 cases, including 87 *stx*$_{2f}$ STEC isolates, were reported between 2008 and 2011. Most *stx*$_{2f}$ strains belonged to the serogroups O63:H6 (n=47, 54%), O113:H6 (n=12, 14%) and O125:H6 (n=12, 14%). Of the 87 *stx*$_{2f}$ isolates, 84 (97%) harboured the *E. coli* attaching and effacing (*eae*) gene, but not the enterohaemorrhagic *E. coli* haemolysin (*hly*) gene. *stx*$_{2f}$ STEC infections show milder symptoms and a less severe clinical course than STEC O157 infections. Almost all infections with *stx*$_{2f}$ (n=83, 95%) occurred between June and December, compared to 170/198 (86%) of STEC O157 and 173/264 (66%) of other STEC non-O157. *stx*$_{2f}$ STEC infections in the Netherlands are more common than anticipated, and form a distinct group within STEC with regard to virulence genes and the relatively mild disease.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is an important pathogen worldwide, associated with human illness, most notably diarrhoea, bloody diarrhoea, haemorrhagic colitis, and haemolytic uraemic syndrome (HUS) [1,2]. Ruminants, especially cattle, are considered the main reservoir for STEC, from where it spreads to humans by contaminated food and/or water. A broad range of virulence factors is associated with the severity of STEC infection [3]. Shiga toxin is an essential factor for the development of severe symptoms like HUS and can be divided into two main types: Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Within both groups, several variants are distinguished. Variant *stx*$_{2f}$ is one of the latest described in the literature, found in *E. coli* strains from pigeons [4-7]. So far, reports of human illness due to *stx*$_{2f}$ STEC are scarce [5,8,9]. *Stx*$_{2f}$ genes were present in only one of 62 isolates of STEC non-O157 cases in the United Kingdom between 1983 and 2000 [10], but was not found in 530 isolates of STEC non-O157 cases in Germany in the period from 1996 to 2000 [11]. Prager et al. [12] presented data from 32 *stx*$_{2f}$ STEC cases identified between 2004 and 2007 in Germany, suggesting that this might be an emerging pathogen. In Japan, between 1996 and 2006, 24 cases with a rare STEC non-O157 serogroup infection were tested for *stx*$_{2f}$, yielding two cases [9]. Furthermore, two relatives of these cases were found to be asymptomatic *stx*$_{2f}$ STEC carriers. During a multi-centre study in the Netherlands (2005–2006), isolates of 21 STEC cases were tested for *stx*$_{2f}$ of which three (14%) tested positive, which, at that time, was already higher than expected based on the previous international reports [13].

In the Netherlands, STEC isolates are submitted to the National Institute for Public Health and the Environment (RIVM) for confirmation and further typing. Since 2007, submitted strains have been routinely tested for *stx*$_{2f}$. This lead to the observation that *stx*$_{2f}$ STEC was relatively common. The question was raised whether *stx*$_{2f}$ STEC cases had distinct clinical and epidemiological characteristics compared to other STEC cases.

Methods

Since January 1999, an enhanced surveillance of STEC O157 has been implemented in the Netherlands. STEC became notifiable in the same year, effectively being STEC O157. In 2007, STEC non-O157 has been added to the enhanced surveillance, which effectively started running in 2008. The notifications of STEC non-O157 do not cover the whole country, as only a fraction of the

laboratories use molecular methods for the detection of all STEC, although the number of laboratories capable of doing this is rising. All medical microbiological laboratories in the Netherlands have to report a positive result for STEC to the local public health service. In addition, they can voluntarily send up to five isolates per patient to the RIVM for confirmation, free of charge. Putative STEC colonies are tested by polymerase chain reaction (PCR) for the presence of the Shiga toxin 1 (stx_1), Shiga toxin 2 (stx_2), *E. coli* attaching and effacing (*eae*) and enterohaemorrhagic *E. coli* haemolysin (*hly*) genes using primers as described by Paton et al. [14]. The presence of stx_{2f} is tested with the PCR method as described by Schmidt et al. [4]. If *stx*-positive colonies are detected, O- and H-typing are performed [15,16].

The regional public health services gather information about age, sex, symptoms and date of illness onset of each case as part of the notification. In the enhanced surveillance, regional public health services are also asked to complete a more elaborate questionnaire together with the case about the clinical manifestation and possible risk factors, such as food consumption, and outdoor activities in the week before date of onset. Cases with a STEC infection and an isolate confirmed and typed at the RIVM between January 2008 and December 2011 were included in the current analysis. In this period, one national outbreak of STEC O157 (n=19 cases) and the German outbreak of STEC O104 (n=11 cases) were identified [17,18]. Cases linked to these outbreaks were excluded.

Since 2008, a control survey in the general population has been added in the Netherlands; three times a year, a questionnaire intended for all age groups is sent to a sample of the general population, containing similar questions as used for cases with notifiable gastroenteritis pathogens and respiratory infections about health and underlying diseases, food consumption, and outdoor activities. This survey is set up to determine risk factors for these diseases, including trends through the years; the survey can also be helpful in investigations of outbreaks caused by these pathogens and infections, especially when an outbreak is diffuse in space and/or time. Between July 2008 and December 2011, 3,908 control questionnaires were mailed and 1,420 were returned (overall response of 36.3%).

Stx_{2f} STEC was compared with other STEC, divided into STEC O157 and STEC non-O157, regarding O type and presence of other genes, age and symptoms of the cases, and risk factors. Differences were tested using the chi-squared test (with $p<0.05$ considered significant). A similar comparison was done between stx_{2f} STEC cases and controls concerning the risk factors, extended with a logistic regression analysis to calculate adjusted odds ratios.

Results

Between 2008 and 2011, a total of 549 STEC cases were reported for which the STEC could be isolated and typed, resulting in 198 O157 infections and 351 non-O157 infections (Table 1). The steady rise of STEC non-O157 infections over time is most likely due to the increasing number of laboratories using PCR-techniques to identify all STEC infections. A quarter (n=87) of the 351 STEC non-O157 isolates contained the stx_{2f} gene. None of the STEC O157 isolates contained the stx_{2f} variant. Of the 87 stx_{2f} isolates, 84 (97%) harboured the *eae* gene, but not the *hly* gene. The remaining three contained neither *eae* nor *hly*. For the other 264 STEC non-O157 isolates, this was six (2%) with *eae* but not *hly* and 83 (31%) with neither *eae* nor *hly*. All 198 STEC O157 isolates contained *hly* and all but one isolate *eae*.

Table 1

Shiga toxin genes (*stx*) in human STEC non-O157 (n=351) and O157 (n=198) isolates, the Netherlands, 2008–2011

Type	2008	2009	2010	2011	Total
STEC non-O157 total	45	51	81	174	351
Stx_1[a] n(%)	14 (31)	25 (49)	31 (38)	70 (40)	140 (40)
Stx_2[b] n(%)	16 (36)	11 (22)	16 (20)	41 (24)	84 (24)
Stx_1+stx_2 n(%)	5 (11)	4 (8)	12 (15)	19 (11)	40 (11)
Stx_{2f}[c] n(%)	10 (22)	11 (22)	22 (27)	44 (25)	87 (25)
STEC O157 total	45	38	50	65	198
Stx_1[a] n(%)	0 (0)	1 (3)	1 (2)	0 (0)	2 (1)
Stx_2[b] n(%)	18 (40)	17 (45)	17 (34)	19 (29)	71 (36)
Stx_1+stx_2 n(%)	27 (60)	20 (53)	32 (64)	46 (71)	125 (63)
Stx_{2f}[c] n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

STEC: Shiga toxin-producing *Escherichia coli*.

[a] Stx_1 was found but not stx_2 in these isolates.

[b] Stx_2 was found but not stx_1 in these isolates.

[c] In these isolates stx_{2f} was found but not stx_1 or other stx_2.

Besides STEC O-non-typable, 65 different STEC non-O157 O-types were found between 2008 and 2011, of which 11 O-types were also seen with the stx_{2f} gene (Table 2). Especially O63:H6 was related to stx_{2f}, followed by O113:H6 and O125:H6.

Ninety five per cent of the stx_{2f} STEC infections (83/87) occurred between June and December (Figure). STEC O157 infections also show a seasonal trend, although somewhat less pronounced, with 170/198 (86%) between June and December. No clear seasonal trend is seen in the other STEC non-O157 infections (June–December: 173/264; 66%).

The median age of cases with a stx_{2f} STEC infection was 31 years, compared to 28 years for cases with another STEC non-O157 infection and 21 years for cases with an STEC O157 infection (Table 3). The differences in median age and age distribution were not statistically significant, although O157 cases tended to be younger. Cases with an stx_{2f} STEC infection had significantly less frequently blood in the stool than STEC O157 cases ($p<0.0001$) and a smaller proportion had problems with low production of urine during the infection than for cases of STEC O157 ($p<0.0001$) and non-O157 ($p=0.03$) (Table 3). Furthermore, stx_{2f} STEC cases had less frequently stomach ache ($p<0.0001$), were reported with no HUS ($p=0.049$) and were less frequently hospitalised ($p=0.001$) compared to STEC O157 cases. The percentages of stx_{2f} STEC cases with such characteristics were however similar to those in the other non-O157 STEC cases. None of the stx_{2f} STEC cases died due to the infection, compared to one of 224 other STEC non-O157 cases (0.4%) and two of 186 STEC O157 cases (1%) for which the information was known.

Cases with a stx_{2f} STEC infection less frequently reported having had contact with someone with gastrointestinal complaints in the week before illness onset than the other STEC non-O157 ($p=0.030$) and STEC O157 cases ($p=0.046$; Table 4). On the other hand, the stx_{2f} STEC cases more frequently reported having eaten dairy products made of raw milk ($p=0.010$) or bean sprouts ($p=0.018$) than STEC O157 cases. The difference in consumption of bean sprouts between stx_{2f} cases and other STEC non-O157 cases was close to significance (0.055). Bean sprouts were also eaten more often by stx_{2f} cases compared to the controls; the odds ratio for consumption of bean sprouts was 2.3 (95% confidence interval: 1.1–5.1), adjusted for age, sex and urbanisation level. No specific questions about contact with birds were included in the questionnaire, except for owning poultry, but it could be reported in open questions addressing contact with animals. None of the 31 stx_{2f} STEC cases reported contact with birds, compared to six (6%) and nine (7%) of the other 98 STEC non-O157 and the 135 STEC O157 cases for which the information was known, respectively.

Table 2

STEC serotypes (n=11) found to contain the Shiga toxin 2f gene (stx_{2f}), the Netherlands, 2008–2011

Serotype	With stx_{2f}/O-type	Per cent of stx_{2f} (n=87)
O2	2/4	2
O2:H-	1/1	1
O2:H6	1/2	1
O2:H29	0/1	0
O16:H5	1/1	1
O35:H19	1/1	1
O63:H6	47/47	54
O73:H18	1/2	1
O96:H7	1/1	1
O101:H-	1/3	1
O113	15/30	17
O113:H-	2/3	2
O113:H4	0/7	0
O113:H6	12/12	14
O113:H7	1/1	1
O113:H21	0/7	0
O121:H5	1/1	1
O125:H6	12/12	14
O132:H34	4/6	5
ONT:H6	1/2	1
Total	**87/110**	**100**

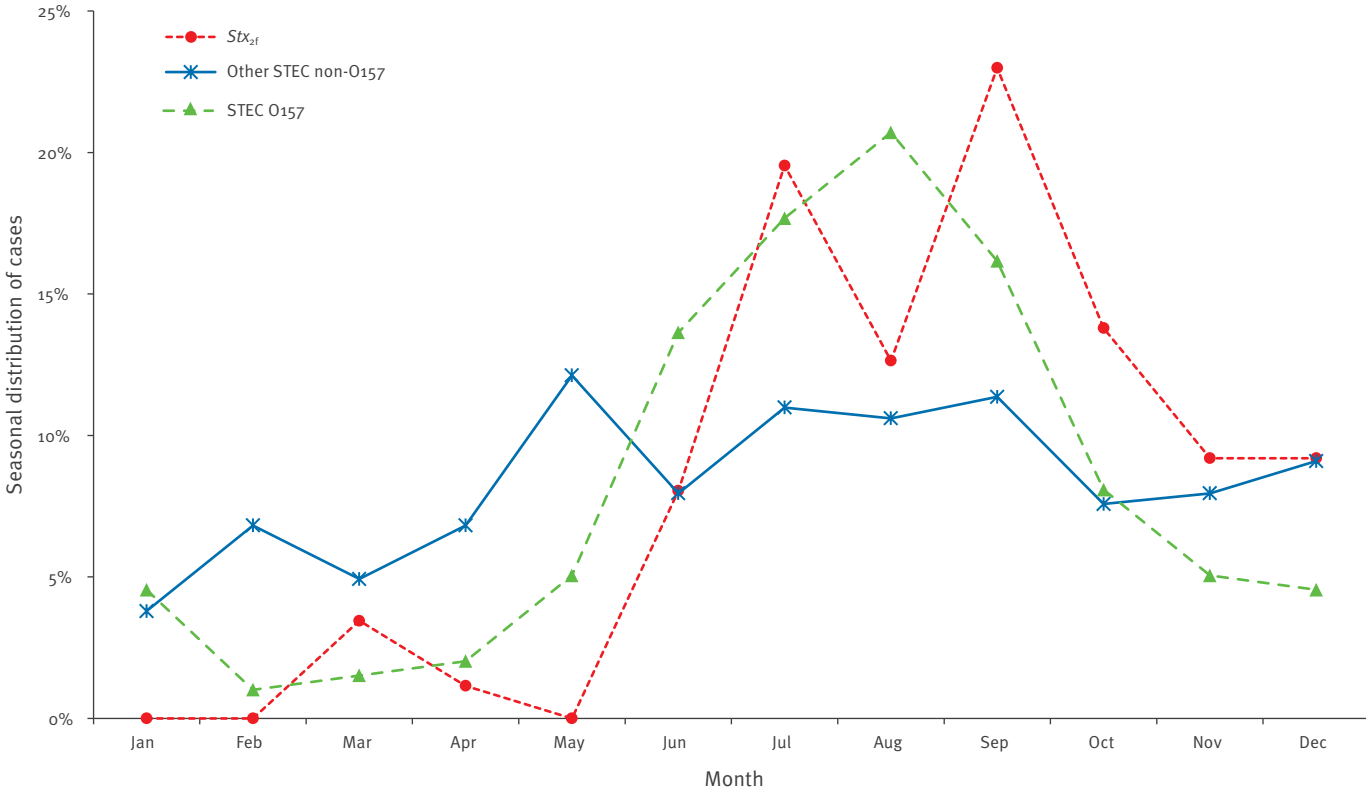
STEC: Shiga toxin-producing *Escherichia coli*.

Discussion

Between 2008 and 2011, stx_{2f} STEC infections comprised 25% of all STEC non-O157 infections in this period and 16% of all STEC isolated. Excluding the laboratories unable to detect all STEC infections, stx_{2f} STEC infections constitute 20% of all STEC infections. This is clearly higher than reported before [9,10,12]. Within a Dutch multi-centre study between 2005 and 2006, three (14%) of 21 STEC cases tested, were positive for stx_{2f}, which was already higher than earlier reports [13]. As these studies all were done before 2008, the relative high percentage in the present study could be a sign that stx_{2f} STEC is emerging. In Belgium, the percentage stx_{2f} STEC was 13% (all STEC) or 17% (STEC non-O157) over the period from 2008 to 2010 [19]. The low frequency of internationally reported human stx_{2f} STEC infections may be due to the mild course of the disease and due to underdiagnosis, as several STEC assays targeting stx (genes) are not capable of detecting the stx_{2f} variant. For example, standard PCR assays and GeneDisc real-time PCR do not detect stx_{2f} [20-22], but requires a specific primer/probe design [21]. Beutin et al. [23] tested two enzyme immunoassays, of which P1-glycoprotein-enzyme immunoassay (EIA) could not and Ridascreen-EIA could detect stx_{2f}. The seemingly mild disease caused by stx_{2f} STEC infections does not stimulate adjusting the commonly used techniques which, in turn, enhances underdiagnosis and underreporting. To be certain however, that stx_{2f} STEC

Figure

Seasonal distribution of *stx*$_{2f}$ STEC (non-O157) cases (n=87), other STEC non-O157 cases (n=264) and STEC O157 cases (n=198), the Netherlands, 2008–2011



STEC: Shiga toxin-producing *Escherichia coli*; *stx*$_{2f}$: Shiga-toxin 2f gene.

Table 3

Reported clinical data of the Shiga toxin-producing *Escherichia coli* (STEC) cases, the Netherlands, 2008–2011 (n=549)

Characteristic	Stx2f STEC non-O157 n/N[a] (%)	Other STEC non-O157 n/N[a] (%)	STEC O157 n/N[a] (%)
Median age in years (minimum–maximum)	31 (0–90)	28 (0–92)	21 (0–85)
Age groups			
0–4 years	17/87 (20)	41/264 (16)	40/198 (20)
5–19 years	15/87 (17)	58/264 (22)	52/198 (26)
20–39 years	17/87 (20)	63/264 (24)	46/198 (23)
40–59 years	20/87 (23)	46/264 (17)	30/198 (15)
>60 years	18/87 (21)	56/264 (21)	30/198 (15)
Sex, % male	41/85 (48)	99/259 (38)	75/198 (38)
Diarrhoea	53/60 (88)	156/179 (87)	164/173 (95)
Stomach ache	36/60 (60)	126/178 (71)	151/172 (88)[b]
Blood in stool	9/60 (15)	47/178 (26)	132/173 (76)[b]
Less urine production	2/59 (3)	24/176 (14)[b]	49/170 (29)[b]
HUS	0/63 (0)	4/191 (2)	11/182 (6)[b]
Hospitalisation	9/58 (16)	29/175 (17)	73/177 (41)[b]
Deceased	0/72 (0)	1/224 (0)	2/186 (1)

HUS: haemolytic uraemic syndrome.
In the Table the denominators of the fractions vary because the information in question was not available from all cases.

[a] Fractions and the resulting percentages are given unless otherwise specified.
[b] Significantly different from *stx*$_{2f}$ cases (p<0.05).

Table 4

Risk factors as reported by the STEC cases (n=417)[a] and community controls (n=1,396)[b], the Netherlands, 2008–2011

Risk factor	Stx_{2f} STEC non-O157 n/N (%)	Other STEC non-O157 n/N (%)	STEC O157 n/N (%)	Controls n/N (%)
Contact with ill person	1/31 (3)	19/98 (19)[c]	24/135 (18)[c]	NA
Contact with livestock	8/31 (26)	28/98 (29)	32/135 (24)	254/1,236 (21)
Dairy products of raw milk consumption	7/31 (23)	16/98 (16)	10/135 (7)[c]	285/1,320 (22)
Raw or undercooked meat consumption	12/31 (39)	52/98 (53)	53/135 (39)	545/1,386 (39)
Raw vegetables/salads consumption	23/29 (79)	71/95 (75)	95/133 (71)	1,080/1,348 (80)
Bean sprouts consumption	11/28 (39)	20/94 (21)	24/129 (19)[c]	301/1,338 (2)[c]
Travel abroad	9/57 (16)	39/182 (21)	32/178 (18)	195/1,364 (14)

NA: not available; STEC: Shiga toxin-producing *Escherichia coli*.

In the Table the denominators of the fractions vary because the information in question was not available from all cases or controls.

[a] The number of STEC cases provided is the number of cases with information on at least one of the risk factors listed in the table available (417 of 549 total STEC cases).

[b] The number of community controls provided is the number of controls with information on at least one of the risk factors listed in the table available (1,396 of 1,420 total controls).

[c] Significantly different from stx_{2f} cases ($p<0.05$).

infections are in fact generally mild, more testing and research is needed.

Almost all stx_{2f} isolates possessed the *eae* gene, which was also reported for the stx_{2f} isolates found in pigeons [7] and in earlier reports of human stx_{2f} STEC infections [9,12]. None of the stx_{2f} isolates contained the *hly* gene, as was also reported by Prager et al. [12] and Seto et al. [9]. The combination of the presence of the *eae* gene but absence of the *hly* gene is rarely seen in the other (non-stx_{2f}) STEC non-O157 infections and not seen at all in STEC O157 infections within the Dutch STEC surveillance. The absence of a finding in this study of a single isolate with a stx_{2f} gene together with a stx_1 gene and the fact that this combination is not reported in the literature so far suggests that stx_{2f} isolates form a distinct group within the STEC infections.

Escherichia albertii has recently been identified as *eae*-positive *Escherichia*, including stx_{2f} strains [24-27]. The stx_{2f} strains were isolated from a patient with diarrhoea and from a healthy crow-like bird [27]. Due to those characteristics, *E. albertii* strains might be misidentified as enterohaemorrhagic *E. coli* (EHEC) or STEC. *E. albertii* and *E. coli* are strongly related and are difficult to discriminate based on 16S sequence (data not shown). Nine isolates in the present study were specifically tested for the inability to ferment lactose, which is a phenotypic trait discriminating *E. albertii* from *E. coli* [27]. Based on this biochemical test all nine tested isolates belonging to the present study appeared to be *E. coli*. However, this does not entirely exclude the possibility that part of the remaining isolates is *E. albertii* instead of *E. coli*.

In the period from 2008 to 2011, the stx_{2f} isolates analysed in this study, belonged to 11 O-types, with four O-types accounting for 87%. O63:H6, appears to be most often associated with stx_{2f}, based on this study and previous reports [9,12,13]. Also, the association with serotype O132:H34 has been reported before [12]. O113:H6, O125:H6 and the more rare O-types of the stx_{2f} isolates have not been related to stx_{2f} before. None of the serogroups found in the current study have been reported in pigeons or other birds [4,7].

Preliminary results from a molecular risk assessment study (data not shown) included five O63 strains and these were all found to harbour relatively low numbers of additional STEC virulence genes. In addition, these O63 strains all belonged to phylogroup B2 while the majority of the other STEC tested belonged to phylogroup B1. There are indications that strains belonging to different phylogroups have different ecological niches and life-history traits. Phylogroup A and B1 strains appear to be generalists, able to occupy a broad range of vertebrate hosts, while B2 and D strains are more commonly isolated from birds and mammals [28]. Phylogroup B2 strains are considered to mainly host adapted *E. coli* with longer persistence in hosts than strains belonging to other phylogroups. In addition, phylogroup B2 generally harbour extra-intestinal virulence traits at higher frequency [29].

No significant difference in age distribution was found between stx_{2f} STEC cases and other STEC cases. Twenty per cent of the Dutch stx_{2f} STEC cases were four years or younger, while 79% of the German stx_{2f} STEC cases reported by Prager et al. [12] were in this age group. The course of a stx_{2f} STEC infection was less severe compared to other STEC infections, especially STEC O157 infections. The reason of this less severe course is unknown, but one could hypothesise that stx_{2f} toxin is less toxic or produced in lower quantities than the other Shiga toxin variants. The putative presence – or

absence – of other virulence genes not determined in this study could also be involved.

This is the first report of possible risk factors and sources for stx_{2f} STEC. The risk factor analysis revealed that person-to-person transmission seems to be less relevant in stx_{2f} STEC infections as compared to other STEC infections. However, the mild course of the infection might mask shedding (contact) persons, which are then not diagnosed nor reported by case patients. stx_{2f} STEC cases reported eating dairy products made of raw milk, and bean sprouts more often than STEC O157 cases.

To confirm whether stx_{2f} STEC is partially foodborne, food products incriminated in our case–control study, or, when occurring, outbreak investigations could be tested for stx_{2f} STEC. When foodborne or other non-human stx_{2f} STEC are found, comparison with human isolates of the same serotype with for example pulsed-field gel electrophoresis (PFGE) or sequence-based typing techniques, could help to further elucidate sources and reservoirs of stx_{2f} STEC and determine the genetic heterogeneity within these serotypes. Beutin et al. [30] tested 219 STEC strains from meat, milk and cheese samples for serotype and genetic variants of Shiga toxins in the period from 2005 to 2006. None of these strains tested positive for stx_{2f}.

Stx_{2f} STEC was first detected in the gastrointestinal tract of apparently healthy pigeons in Italy and Germany [4,31]. With the relative high faecal carriage of stx_{2f} STEC in pigeons, ranging from six to 16%, pigeons were considered as a reservoir [7]. However, the present study did not point in the direction of pigeons or other birds, as none of the serogroups found have been reported in avian species. In addition, none of the stx_{2f} STEC cases reported contact with birds. It should be noted that as the data about risk factors were available for only a part (31/87; 36%) of the stx_{2f} STEC cases, less strong associations might have been missed due to lack of power.

In conclusion, human stx_{2f} STEC infections are more common than anticipated in the Netherlands, with an estimated 20% of all STEC infections constituting the stx_{2f} gene. Stx_{2f} STEC form a clinically and microbiologically distinct group within STEC, mostly harbouring the *eae*, but lacking the *hly* gene and not seen together with other *stx* genes or in STEC O157. Stx_{2f} STEC infections appear to be relatively mild compared to other STEC infections, especially STEC O157. The present study could not confirm exposure to pigeons or other birds as source for infections, but alternatively found, although not very strong, associations with raw dairy products and bean sprout consumption. To further explore such associations, more research would be needed, also using additional diagnostic and typing methods. The trend in stx_{2f} will be further monitored in the coming years and will also allow more powerful case–control analyses.

Conflict of interest

None declared.

Authors' contributions

IF coordinated the collection of data, analysed and interpreted the data and drafted the manuscript. KvdZ confirmed and typed the isolates, and participated in editing the manuscript. TS and MK-S developed/adapted the PCR method for detecting STEC, and participated in editing the manuscript. EF participated in the interpretation of the data and editing the manuscript. YvD previously coordinated the collection of data, and YvD and WvP participated in the interpretation of the data and editing the manuscript. All authors read and approved the final manuscript.

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